Activity-dependent changes in neuropsin gene expression in the hippocampus implies an involvement of neuropsin in neural plasticity. Since the deduced amino acid sequence of the gene contained the complete triplet (His-Asp-Ser) of the serine protease domain, the protein was postulated to have proteolytic activity. Recombinant full-length neuropsin produced in the baculovirus/insect cell system was enzymatically inactive but was readily converted to active enzyme by endoprotease processing. The activation processing of prototype neuropsin involved the specific cleavage of the Lys32-Ine23 bond near its N terminus. Native neuropsin that was purified with a purity of 1,100-fold from mouse brain had enzymatic characteristics identical to those of active-type recombinant neuropsin. Both brain and recombinant neuropsin had amidolytic activities cleaving Arg-X and Lys-X bonds in the synthetic chromogenic substrates, and the highest specific activity was found against Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide. The active-type recombinant neuropsin effectively cleaved fibronectin, an extracellular matrix protein. Taken together, these results indicate that this protease, which is enzymatically novel, has significant limbic effects by changing the extracellular matrix environment.

Some proteases have been suggested to be related to neural cell dynamics in such processes as cell death, migration, cell-to-cell adhesion and de-adhesion, process elongation, pathfinding, and axonal rearrangement (1–5). These phenomena have been investigated by supplying known proteases involved in blood coagulation, fibrinolysis, or digestion to neural cell cultures. However, the observations that the proteases are mainly localized in and released from non-neural cells do not support all of such neural effects (5–7). Thus, we postulated that neurons themselves may produce and release their own proteases (8). These results and the observation that its mRNA showed marked activity-dependent changes caused by plasticity-inducible stimuli are suggestive of some neural effects in limbic plasticity (8, 9). However, it is still not known whether NP protein has enzyme activity as suggested by the deduced amino acid sequence (8). We postulated that the enzyme activity might be a molecular basis for the physiological responses induced by various stimuli. Therefore, in the present study, we examined whether recombinant NP (r-NP) and brain NP had proteolytic activity against synthetic and natural substrates.

EXPERIMENTAL PROCEDURES

Materials—Mono S, Sepharose 2B, CNBr-activated Sepharose 4B and CL-6B, Superdex 75HR, Superose 12, Resource S, Protein G-Sepharose were from Amersham Pharmacia Biotech. Silver staining kits were from Bio-Rad. Diisopropyl fluorophosphate (DFP), benzamidine, bestatin, soybean trypsin inhibitor, human plasma thrombin (EC 3.4.4.33), and TNN-FH insect cell medium were purchased from Sigma. [1,3-3H]DFP was from NEN Life Science Products. D-Pro-Phe-Arg-pNA, N-Val-Leu-Arg-pNA, and N-Val-Leu-Lys-pNA were from Daiichi Pure Chemicals (Tokyo, Japan). N-Bz-Lys-pNA, dimethyl sulfoxide, apro- tin, protease 1 (Achromobacter lyticus, EC 3.4.21.50), trypsin (EC 3.4.21.4), and polyethylene glycol 20,000 were from Wako Pure Chemical Inc. (Osaka, Japan). Bz-Tyr-pNA, L-Leu-pNA, Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide, Boc-Leu-Val-Pro-Arg-MCA, Boc-Leu-Pro-Arg-MCA, Boc-Leu-Arg-Arg-MCA, and Boc-Leu-Thr-Arg-MCA were from Wako Pure Chemicals (Osaka, Japan). Fetal bovine serum and (4-amidinophenyl)methane-sulfonyl 1-fluoride were from Boehringer Mannheim (Germany). Horseradish peroxidase-labeled goat anti-rat IgG was purchased from Cap- pell. All other reagents used were of analytical grade.

Cloning of Full-length NP cDNA into Baculovirus—The 866-base pair NP3 cDNA was cloned into the Not I site of the pVL1392 transfer vector to create plasmid pVL1392/NP3 (Fig. 1) (8). Plasmid DNA was transferred into the AcNPV genome by homologous recombination so that SF21 cells were transfected with vector transfer and AcNPV DNA. The presence of the NP3 cDNA was confirmed by dot blot

Neuropsin (NP)3 was cloned from the mouse brain and was shown to be localized in mouse hippocampal pyramidal neurons (8). These results and the observation that its mRNA showed marked activity-dependent changes caused by plasticity-inducible stimuli are suggestive of some neural effects in limbic plasticity (8, 9). However, it is still not known whether NP protein has enzyme activity as suggested by the deduced amino acid sequence (8). We postulated that the enzyme activity might be a molecular basis for the physiological responses induced by various stimuli. Therefore, in the present study, we examined whether recombinant NP (r-NP) and brain NP had proteolytic activity against synthetic and natural substrates.

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hybridization. Positive virus clones were harvested from the culture medium, and the DNA was characterized by Southern blotting.

Production of r-NP in Insect Cells—Sf21 insect cells were grown at 27 °C in TNNM-FH medium containing 10% heat-inactivated fetal calf serum to a density of 7 × 10^6/cm² tissue culture flask. For infection, cells were infected with recombinant baculovirus at a multiplicity of infection of 1. Three to five days postinfection, the incubation medium was harvested by removing the cells by centrifugation at 500 × g for 10 min at 4 °C. The supernatant was clarified by centrifugation at 4 °C, 25,000 × g, for 1 h and dialyzed against 10 mM HEPES buffer (pH 7.4) containing 0.04% H2O2. The supernatant was then concentrated using Spectrapore Membranes (M, 3500) against polyethylene glycol 20,000, and the supernatant was dialyzed against 10 mM HEPES buffer (pH 7.4). This solution was subjected to anion exchange chromatography using a 6-ml column of Resource S. Bound protein was eluted with a linear gradient of 0 to 1 M NaCl in 50 min at a flow rate of 2 ml/min. NP was eluted at 0.44–0.58 M NaCl. For further purification, the protein was concentrated by polyethylene glycol 6000 in 50 ml of NaCl (pH 7.4), and gel-filfiltered with a 10 × 300-mm column of Superdex 75 HR. The recombinant protein samples were then applied to SDS-PAGE, followed by silver staining under both reducing and non-reducing conditions as described below. The expressed recombinant protein had no or only low amylodicy activity, and this was assumed to be pro-NP (r-pro-NP) (Table 1).

Protease 1 (EC 3.4.21.50) Activation of r-Pro-NP by Endoproteases—

To analyze cleavage sites of fibronectin by r-NP, the digestion products were electrophoresed and transferred onto Immobilon membranes. The transferred bands on membranes corresponding to degraded fibronectin were cut out and subjected to N-terminal sequencing as described above.

Immunofluorimetry of a mouse brain NP Affinity gel was prepared using the monoclonal antibody produced as described above. Six mg of mAbB5 was dialyzed against 0.1 M carbonate buffer (pH 9.0) overnight. CNBr-activated Sepharose CL-6B was activated and coupled to mAbB5.

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Production, Purification, and Activation of r-pro-NP—The full murine NP sequence was subcloned into the NotI site of the pVL1392 transfer vector (Fig. 1A) (8). By SDS-PAGE and silver staining, a major 32-kDa product was detected in the medium derived from infected cells (Fig. 1, B and C) but not from uninfected cells (data not shown). The 32-kDa protein was immunoreactive with anti-NP monoclonal antibody (mAbB5) on Western blotting, and this band was therefore presumed to correspond to recombinant NP protein. However, conditioned media from infected and non-infected insect cells both lacked amidolytic activity for various synthetic substrates (Table I). Therefore, we speculated that the 32-kDa protein is a non-active prototype NP protein (r-pro-NP). Conditioned medium from infected insect cells showed high amidolytic activity following the endoprotease processing (fluorescence intensity was 5130 per 1 µl of medium; see details below; see also Figs. 1B and 2 and Table I), whereas the medium from non-infected cells still showed very low amidolytic activity even after endoprotease treatment (60.3 fluorescence intensity per 1 µl of medium).

The culture medium derived from infected insect cells was subjected to anion exchange chromatography in one step to purify r-pro-NP. One major peak of protein eluted by a linear NaCl gradient was collected. As the fractions contained minor cell products, they were pooled and concentrated before being applied to gel chromatography in the second step to remove the minor components. The fractions eluted from gel chromatography were used for assay of amidolytic activity after activation by gel-immobilized protease 1 (Fig. 1B, cf. Fig. 2). A single peak of cleavage activity of Boc-Val-Pro-Arg-MCA was observed in fractions 17–20 (Fig. 1B). To check the purity, SDS-PAGE of the fractions was performed, and proteins were transferred onto nitrocellulose membranes. Fraction 19 showed a single band by SDS-PAGE followed by silver staining and Western blotting with mAbB5 and was thus used as purified r-pro-NP (Fig. 1C).

**RESULTS**

**Production, Purification, and Activation of r-pro-NP**—The full murine NP sequence was subcloned into the NotI site of the pVL1392 transfer vector (Fig. 1A) (8). By SDS-PAGE and silver staining, a major 32-kDa product was detected in the medium derived from infected cells (Fig. 1, B and C) but not from uninfected cells (data not shown). The 32-kDa protein was immunoreactive with anti-NP monoclonal antibody (mAbB5) on Western blotting, and this band was therefore presumed to correspond to recombinant NP protein. However, conditioned media from infected and non-infected insect cells both lacked amidolytic activity for various synthetic substrates (Table I). Therefore, we speculated that the 32-kDa protein is a non-active prototype NP protein (r-pro-NP). Conditioned medium from infected insect cells showed high amidolytic activity following the endoprotease processing (fluorescence intensity was 5130 per 1 µl of medium; see details below; see also Figs. 1B and 2 and Table I), whereas the medium from non-infected cells still showed very low amidolytic activity even after endoprotease treatment (60.3 fluorescence intensity per 1 µl of medium).

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**Table I**

| Substrate                                | Specific activity | r-pro-NP | Brain NP |
|------------------------------------------|-------------------|---------|----------|
| Boc-Val-Pro-Arg-MCA                      | 0.01              | 6.26 (100) | 1.23 (100) |
| Boc-Phe-Ser-Arg-MCA                      | 0                 | 4.88 (78)  | 0.50 (41)   |
| B-Val-Leu-Arg-pNA                       | 0                 | 1.75 (28)  | 0.38 (31)   |
| Boc-Ala-Gly-Pro-Arg-MCA                  | —                 | 1.7 (27)   | —          |
| Boc-Glu-Gly-Arg-MCA                      | —                 | 1.48 (24)  | 0.30 (24)   |
| Boc-Asp-Pro-Arg-MCA                      | 1.41 (22)         | —        | —          |
| Boc-Glu-Gly-Arg-MCA                      | —                 | 1.33 (21)  | —          |
| B-Val-Leu-Lys-pNA                        | —                 | 1.27 (20)  | 0.29 (24)   |
| Boc-Pyr-Glu-Gly-MCA                      | —                 | 1.27 (20)  | 0.34 (28)   |
| Boc-Glu-Lys-Lys-MCA                      | —                 | 1.21 (19)  | 0.27 (22)   |
| Boc-Leu-Thr-Arg-MCA                      | —                 | 1.07 (17)  | —          |
| Boc-Leu-Arg-Arg-MCA                      | —                 | 1.01 (16)  | —          |
| Z-Pro-Phe-Arg-pNA                        | —                 | 0.79 (13)  | —          |
| Boc-Gly-Arg-MCA                          | —                 | 0.71 (11)  | —          |
| Suc-Leu-Leu-Val-Tyr-MCA                  | —                 | 0 (0)     | 0 (0)      |
| Z-Phe-MCA                                | —                 | 0 (0)     | 0 (0)      |
| Bz-Tyr-pNA                               | —                 | 0 (0)     | 0 (0)      |
| Bz-L-Arg-pNA                             | —                 | 0 (0)     | 0 (0)      |
| Bz-L-Leu-pNA                             | —                 | 0 (0)     | 0 (0)      |
| N-Bz-DL-Lys-pNA                          | —                 | 0 (0)     | 0 (0)      |

*One unit of activity was defined as that required to hydrolyze 1 µmol/min chromogen. 
*— not tested. 
Glt, glutaryl. 
Pyr, Pyroglutamyl.
Endoprotease treatment of r-pro-NP induced rapid induction of amidolytic activity by conversion to r-NP (Fig. 2). The enzyme activity in this experiment was observed using Z-Pro-Phe-Arg-MCA, whereas it was cleaved less effectively than Boc-Val-Pro-Arg-MCA because it was not cleaved by protease 1 itself. To examine the time course of the induction of activity, various concentrations of protease 1 were incubated with purified r-pro-NP. The r-pro-NP was processed in a dose-dependent manner after 5 min of incubation with protease 1 (Fig. 2). Longer incubation resulted in the enzyme activity reaching a plateau with almost the same activity at 2, 4, and 16 ng of protease 1 (Fig. 2). No induction of the enzyme activity was observed when protease 1 was omitted (Fig. 2, open squares). Enzyme activity of r-NP was characterized using this activated recombinant protein produced by endoprotease processing of r-pro-NP. The effects of pH on r-NP amidolytic activity were examined using four different buffers as follows: acetate (pH 3.5–5.5), phosphate (pH 5.5–7.5), Tris-HCl (pH 7.0–9.0), and carbonate buffer (pH 9.0–10.0). As shown in Fig. 3, the pH optimum for the enzyme activity was around pH 8.0.

To confirm that r-pro-NP is a zymogen of r-NP, we performed N-terminal amino acid sequencing of both r-pro-NP and r-NP. The sequenced N-terminal peptide of r-NP was comprised of 7 amino acids and started at Ile33, 33 amino acids downstream of the deduced amino acid sequence of the NP3 cDNA (data not shown). On the other hand, the N terminus of r-pro-NP was blocked and not sequenced. To identify the N-terminal amino acid of r-pro-NP, the difference of molecular weight between r-NP and r-pro-NP was analyzed by matrix-assisted laser/desorption ionization time-of-flight mass spectrometry (MALDI-TOFMS). MALDI-TOFMS revealed that the molecular masses of r-pro-NP and r-NP were 26.613 and 26.229 kDa, respectively, and thus r-NP was 384 daltons less than r-pro-NP. This difference was suggested to be caused by an N-terminal peptide of r-pro-NP composed of 4 amino acid residues, Gln28-Lys32 (calculated 4 Mw = 400). Some unknown modification(s), probably cyclization, of the N-terminal Gln of r-pro-NP might have been responsible for the inability to determine its N-terminal sequence. Hydrophobicity plot analysis of the deduced amino acid sequence of the NP3 cDNA showed that the peptide from Met1-Ala28 is highly hydrophobic (16), and this was postulated to be the signal sequence. Therefore, we concluded that secreted r-pro-NP from infected insect cells undergoes activation by conversion to r-NP at the Lys32-Ile33 bond as shown in the model (Fig. 4).

As described above, the molecular mass of r-pro-NP was determined to be 26.6 kDa by MALDI-TOFMS analysis. This is in good agreement with the calculated molecular mass (25.5 kDa) from the amino acid content deduced from cDNA (8). The molecular mass was estimated by SDS-PAGE under reducing and non-reducing conditions as 32 (Fig. 1C) and 26 kDa (Fig. 7B), respectively, and by Superose 12 size exclusion chromatography at 29 kDa, suggesting that the enzyme exists as a monomer.

**Substrates and Inhibitors of r-NP**—Table I shows amidolytic activities of r-pro-NP and r-NP on various synthetic substrates. The highest amidolytic activity was observed toward Boc-Val-Pro-Arg-MCA, a substrate of α-thrombin. Boc-Phe-Ser-Arg-MCA, which is a substrate of trypsin, was also a good substrate for r-NP. The effects of various protease inhibitors on amidolytic activity of r-NP are shown in Table II. The enzyme activities were measured strongly inhibited by low molecular weight protease inhibitors that bind to His and Ser residues in the active centers of serine proteases. DFP, leupeptin, and (4-aminophenyl)methanesulfonyl fluoride belong to this group. Inhibition was also seen with benzamidine and antipain. Both pepstatin A, an aspartic protease inhibitor, and E-64, a cysteine protease inhibitor, had no or only slight effects on r-NP activity. In addition, the divalent cations Ca2+ and Mg2+ and metal ion chelators had little effect on r-NP activity. Thus, NP was categorized as a serine protease and not an aspartic, cysteine, or metalloprotease.

Classified inhibitors were applied to define the r-NP activity. Specific low molecular weight inhibitors of chymotrypsin and trypsin, chymostatin and aprotinin markedly inhibited the amidolytic activity of r-NP. However, high molecular weight inhibitors of serine proteases did not significantly inhibit the enzyme activity (Table II). Hence, NP has a similar catalytic center to trypsin and chymotrypsin, but the substrate specificity due to the structure of the active site might be very different from those of these enzymes (see “Discussion”).

**Platelet Aggregation Activity of r-NP**—As the r-NP favorably cleaved Boc-Val-Pro-Arg-MCA, a synthetic substrate of α-thrombin, thrombin-like blood coagulation activity was analyzed. Although 0.1 and 0.5 units of thrombin, used as a positive control, had strong platelet aggregation activity, no activ-
ity was found in r-NP at any concentration tested (0.1, 1, and 2 units; data not shown). These results suggested that r-NP does not process thrombin receptor protein (present results and see Refs. 17 and 18).

Degradation of Fibronectin by r-NP, but Not by r-pro-NP—No or only weak proteolytic activities of r-NP against gelatin and collagen types I, III, IV, and VI were detected by zymography (data not shown). Strong proteolytic activity of r-NP was found against fibronectin, an extracellular matrix protein, which is widely distributed in the nervous system. Human plasma fibronectin is composed mainly of a 440-kDa dimer with lesser but variable amounts of lower molecular weight forms, when analyzed under non-reducing conditions (lane 1 in Fig. 5A, A and B). Upon incubation with r-NP, the molecular mass of fibronectin gradually decreased from the 440-kDa dimer to 220-, 200-, and 170-kDa monomers (Fig. 5A, lanes 2–6). The 220-kDa fragment, which is the monomeric form of fibronectin (19), appeared initially, followed by the 200- and 170-kDa degradation products in a time-dependent manner. Furie and Rifkin (20) demonstrated that the interchain disulfide bridges of the dimerized fibronectin are present very close to the C terminus. Together with this finding, our results indicate that r-NP-mediated cleavage might occur initially near the C-terminal region.

**Table II**

| Inhibitor             | Concentration | % Inhibition |
|----------------------|---------------|--------------|
| Low molecular weight inhibitors |               |              |
| DFP                  | 0.01          | 5            |
|                      | 0.1           | 16           |
|                      | 1             | 80           |
|                      | 10            | 100          |
| Leupeptin            | 0.01          | 75           |
|                      | 0.1           | 100          |
| APMSF\(^a\)          | 0.1           | 0            |
|                      | 0.3           | 19           |
|                      | 1             | 61           |
|                      | 3             | 87           |
| Benzanidin           | 0.1           | 10           |
|                      | 1             | 56           |
| Antipain             | 0.01          | 75           |
|                      | 0.1           | 95           |
| Chymostatin          | 0.01          | 45           |
|                      | 0.1           | 80           |
|                      | 10            | 100          |
| Aprotinin            | 0.01          | 5            |
|                      | 0.1           | 65           |
|                      | 10            | 84           |
| Pepstatin A          | 0.01          | 0            |
|                      | 0.1           | 0            |
| E-64                 | 0.01          | 0            |
|                      | 0.1           | 15           |
| High molecular weight inhibitors |       |              |
| Human a1-antitrypsin | 2             | 14           |
|                      | 10            | 20           |
| Human a1-antichymotrypsin | 0.4  | 7            |
|                      | 2             | 16           |
| Trypsin inhibitor    | 0.01 (mg/ml)  | 0            |

\(^a\) APMSF, (4-amidinophenyl)methanesulfonyl 1-fluoride.
Next, we performed N-terminal amino acid sequencing of the 200- and 170-kDa degradation products and found that these degradation products started at the same site, Ala291. Therefore, r-NP cleaves specific sites of fibronectin as follows: 1) the C terminus, and 2) a site between the fifth and sixth fibronectin type I repeats (FnI). The five FnI repeats in the N terminus are lost following r-NP treatment for 2 h or more (Fig. 5, A and C). No further degradation was observed with incubation up to 6 h (Fig. 5A). Thus, the degradation step of fibronectin was very specific and might be important for physiology of cells because the N-terminal five FnI repeats contain important functional sites (Fig. 5C, Ref. 21; see also “Discussion”).

Partial Purification of Mouse Brain NP—We focused on enzyme characterization of native NP. For this purpose, brain NP from mouse brain homogenate was partially purified (Table III). One-step immunoaffinity chromatography after detergent solubilization of brain lysate resulted in purification with a 1,100-fold purity and a 72.7% recovery with a major band on SDS-PAGE visualized by silver staining (Fig. 6A, asterisks in fractions 3 and 4; Table III). The amidolytic activity measured with Boc-Val-Pro-Arg-MCA as a substrate was eluted in fractions 3 and 4 (Fig. 6B). Western blotting analysis showed a dense band of 32 kDa in fractions 3 and 4 (Fig. 6C). A faint band of 30 kDa in fractions 3 was considered to be a degradation product of NP because it showed immunoreactivity. SDS-PAGE of [3H]-labeled DFP-bound protein originating from fractions 3 and 4 followed by autoradiography showed a single band of approximately 32 kDa (Fig. 6D). A faint band of brain NP was also detected by [3H]DFP autoradiography. Aliquots of 10 μl of partially purified brain NP (NP, fraction 3) and 2 ng of r-NP were electrophoresed.

| Table III | Purification of NP from mouse brain |
|-----------|----------------------------------|
| **Procedure** | **Volume** | **Protein** | **Total activity** | **Specific activity** | **Purity (integers)** | **Yield** |
| Brain homogenate | 19.7 | 220 | 0.11 | 0.0005 | 1 | 100 |
| Immunoaffinity chromatography | 0.6 | 0.14 | 0.08 | 0.55 | 1100 | 72.7 |

*a One unit of activity was defined as that required to hydrolyze 1 μmol/min chromogen.

Partial purification of NP. The mAbB5-coupled affinity gel (see “Experimental Procedures”) was used for purification of NP from brain. Fraction size was 1 ml each. A, electrophoresis of aliquots (10 μl) of eluates was performed in a 12.5% gel followed by silver staining and Western blotting. Lane 1, eluate fraction; lane 2, pool of wash; and lanes 3–5, eluted 1-ml fractions. B, enzyme activity of each fraction measured with Boc-Val-Pro-Arg-MCA as a substrate. Note that cleavage activity for the chromogenic substrate was found in fractions 3 and 4. C, Western blotting analysis of each fraction demonstrated that NP was present in fractions 3 and 4. D, autoradiography of [3H]DFP bound to partially purified NP and r-NP. A single band of approximately 32 kDa was detected by [3H]DFP autoradiography. Aliquots of 0.1 ml of partially purified brain NP (NP, fraction 3) and 2 ng of r-NP were electrophoresed.

**Table IV** Comparison of $K_m$ values between brain NP and r-NP

| Substrate | r-NP | brain NP |
|-----------|------|----------|
| Boc-Val-Pro-Arg-MCA | 270 (μM) | 300 (μM) |
| Boc-Phe-Ser-Arg-MCA | 500 | 540 |
| D-Val-Leu-Arg-pNA | 230 | 280 |
| Boc-Asp-Pro-Arg-MCA | 398 | — |

—, not tested.

immunoprecipitation and immunoblotting analysis. Major and a minor weak bands corresponding to brain NP and its degradation product, respectively, were detected in the soluble fraction (A), whereas no band was detected in the Triton X-100-soluble fraction (B and C). A faint band of brain NP was also detectable in the cytoskeleton-rich fraction (D).

**DISCUSSION**

It has been suggested that neural plasticity is based on the actions of a variety of proteases and their inhibitors (3, 22–25). Various neurological stimuli induce tissue-type plasminogen activator (tPA) mRNA in the brain, and it has been shown to be involved in seizures, kindling, and neural degeneration (5, 23, 26). It was also suggested that tPA might be related to repulsion of neural outgrowth, because inhibitors of tPA increase neurite outgrowth in sympathetic ganglion cells (27). Such induction of neurite retraction on the cultured central and peripheral neurons was also found for thrombin (4, 28–31). However, these proteases have been shown to be involved only in gial to neuronal interactions in the developing brain (1, 5,
Brain NP which was affinity purified from brain homogenate had enzymatic properties identical to those of r-NP. The enzyme preferentially hydrolyzes Arg-X bonds and, to a lesser extent, Lys-X bonds. In addition, the tripeptide MCA substrate Z-Pro-Phe-Arg-MCA, but not the dipeptide MCA substrate Z-Phe-Arg-MCA, was cleaved by the enzyme, and thus at least four binding sites (S_4, S_3, S_2, and S_1) seem to be a prerequisite for hydrolysis (33, 34). The enzyme therefore appears to contain multiple amino acid side chain binding sites in its active site. The S_3 subsite appears to favor hydrophobic (Val, Phe) side chains, judging from the higher specific activities of NP for Boc-Val-Pro-Arg-MCA and Boc-Phe-Ser-Arg-MCA than for Boc-Asp-Pro-Arg-MCA (see Table I). The order of specific activities of the three highest activity substrates was Boc-Val-Pro-Arg-MCA > Boc-Phe-Ser-Arg-MCA > d-Val-Leu-Arg-pNA (see Table I). Human thrombin cleaved Boc-Val-Pro-Arg-MCA faster than Boc-Phe-Ser-Arg-MCA, but the activity for the latter substrate was only 3% of that for the former (cf. 48–78% in NP, Table I) (35). In addition, porcine kallikrein and plasmin cleaved Boc-Val-Pro-Arg-MCA more slowly than d-Val-Leu-Arg-pNA (35). Thus, the substrate specificity of NP is very different from those of thrombin, tissue kallikrein, and plasmin.

By the subcellular fractionation of mouse brain, NP co-fractionated with the salmine-soluble fraction which is composed of cytoplasm and extracellular soluble components (Fig. 7 and Ref. 15). As the hydrophobic signal sequence is encoded in the NP3 cDNA (8) and the recombinant protein is released into medium from NP3 cDNA-infected insect cells as shown in the present study and NP3 cDNA-transfected neuroblastoma cells (Neuro 2a). Therefore, NP is strongly suggested to be an extracellular protease. Recently, we reported that intraventricular injection of monoclonal antibodies specific to NP into mouse brain reduced the epileptic pattern of electroencephalograms and epileptic behavior (11). As exogenously applied antibodies can hardly pass across the plasma membrane into living cells, the antibodies are thought to modify the activity of extracellular NP. Taken together, these observations suggested that brain NP secreted from hippocampal pyramidal neurons as a zymogen and undergone an activational processing might be involved in neural plasticity-related protease function (present study and Refs. 8, 9, and 11).

Since NP is considered to be a secretory serine protease as described above, the extracellular matrix molecules are good candidates for the physiological substrates of NP. r-NP effectively cleaved fibronectin which is a major extracellular matrix protein expressed in the nervous system (present study and Ref. 37). The specific cleavage by r-NP as shown in the present study might directly affect fibronectin’s functions as a cell adhesion molecule, because the N-terminal 5 FnI domains contain the main fibrin-binding site (21). Such cleavage pattern of fibronectin by r-NP is analogous to that by plasmin but was different from those by thrombin, plasminogen activator, and trypsin (19, 38).

In conclusion, the present study clearly demonstrated characters of r-NP and brain NP as a novel serine protease and also presented that the protease might be involved in neural plasticity by potential modification of the extracellular environments.

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24) On the other hand, proteases relating to intersynaptic connections among neurons to neurons in the matured brain have not been identified. We hypothesized the existence of a novel protease localized in and released from the pyramidal neurons of the hippocampus, and we cloned the NP cDNA from these brain areas as described in our previous reports (8, 9, 32). The NP mRNA was expressed in pyramidal neurons of the hippocampus with the highest density in the brain, and the neural responses of this gene strongly suggested that this protease has important physiological functions in the limbic brain.

Analyses of the deduced amino acid sequence of the NP3 cDNA suggested that this protein has protease activity. Studies were begun to produce a recombinant protein in the baculovirus-insect cell system and to measure the protease activity of the recombinant protein. However, in contrast to our initial assumption, the recombinant protein did not show clear cleavage activity for any synthetic protease substrates examined. The present study, however, clearly demonstrated that recombinant prototype NP (r-pro-NP) secreted from insect cells was processed by endoproteases and was converted to the enzymatically active form (r-NP) as shown in Fig. 4. It is interesting that the activational processing was brought about by the removal of only four N-terminal amino acids.
REFERENCES

1. Monard, D. (1988) Trends Neurosci. 11, 541–544
2. Pittman, R. N., Ivans, J. K., and Buettner, H. M. (1989) J. Neurosci. 9, 4269–4286
3. Liu, Y., Field, R. D., Fitzgerald, S., Festoff, B. W., and Nelson, P. G. (1993) J. Neurobiol. 23, 325–335
4. Suiden, H. S., Stone, S. R., Hemmings, B. A., and Monard, D. (1992) Neuron 8, 363–375
5. Tsirka, S. E., Guandalinis, A., Amaral, D. G., and Strickland, S. (1995) Nature 377, 340–344
6. Dent, M. A. R., Sumi, Y., Morris, R. J., and Seeley, P. J. (1993) Eur. J. Neurosci. 5, 633–647
7. Rao, J. S., Sawana, R., Gokaslan, Z. L., Yung, W. K. A., Goldstein, G. W., and Laterra, J. (1996) J. Neurochem. 66, 1657–1664
8. Chen, Z.-L., Yoshida, S., Kato, K., Momota, Y., Suzuki, J., Tanaka, T., Ito, J., Nishino, H., Aimoto, S., and Shiosaka, S. (1995) Brain Res. 728, 116–120
9. Okabe, A., Momota, Y., Yoshida, S., Hirata, A., Ito, J., Nishino, H., and Shiosaka, S. (1997) J. Biol. Chem. 272, 3074–3079
10. Nishizawa, K., Yano, T., Shibata, M., Ando, S., Saga, S., Takahashi, T., and Inagaki, M. (1991) J. Biol. Chem. 266, 3074–3079
11. Momota, Y., Yoshida, S., Ito, J., Shibata, M., Kato, K., Matsumoto, K., and Shiosaka, S. (1997) Eur. J. Neurosci. 10, 760–764
12. Laemmli, U. K. (1970) Nature 227, 680–685
13. Dammerval, C., Le Guilloux, M., Blaisonseau, J., and de Vienne, D. (1987) Electrophoresis 8, 158–159
14. Wiedelmann, K., Braun, R., and Fitzpatrick, J. (1988) Anal. Biochem. 175, 231–237
15. Yen, S.-H., Kenessey, A., Lee, S.-C., and Dickson, D. W. (1995) J. Neurochem. 65, 2577–2584
16. Kyme, J., and Doolittle, R. F. (1982) J. Mol. Biol. 157, 105–132
17. Wu, T.-K., Hung, D.-T., Wheaton, V. L., and Coughlin, S. R. (1991) Cell 64, 1057–1068
18. Rasmussen, U. B., Gachet, C., Schlesinger, Y., Hanau, D., Uhlmann, P., Van Oevergen-Schilling, E., Pouyssegur, J., Cazeneuve, J. P., and Pavirani, A. (1993) J. Biol. Chem. 268, 14322–14328
19. Quigley, J. P., Gold, L. I., Schwimmer, R., and Sullivan, L. M. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2776–2780
20. Furie, M., and Rifkin, D. B. (1980) J. Biol. Chem. 255, 3134–3140
21. Chothia, C., and Jones, E. Y. (1987) Annu. Rev. Biochem. 56, 823–862
22. Lynch, G., and Baudry, M. (1984) Science 224, 1067–1063
23. Qian, Z., Gilbert, M. E., Colicos, M. A., Kandel, E. R., and Kuhl, D. (1993) Nature 361, 453–457
24. Nelson, P. G., Fields, R. D., and Lie, Y. (1994) Perspect. Dev. Neurobiol. 2, 399–407
25. Frey, U., Muller, M., and Kuhl, D. (1996) J. Neurosci. 15, 2057–2063
26. Twining, S. S. (1994) Crit. Rev. Biochem. Mol. Biol. 29, 315–383
27. Seeds, N. W., Friedman, G., Hayden, S., Thewke, D., Hoffke, S., McGuire, P., and Krystosek, A. (1996) Semin. Neurosci. 8, 405–412
28. Jalink, K., and Moelenaar, W. J. (1992) J. Cell Biol. 118, 411–419
29. Gurwitz, D., and Cunningham, D. D. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 3440–3444
30. Hawkins, R. L., and Seeds, N. W. (1986) Brain Res. 398, 63–70
31. Grand, R. J. A., Graham, P. W., Gallimore, M. J., and Gallimore, P. H. (1989) EMBO J. 8, 2209–2215
32. Suzuki, J., Yoshida, S., Chen, Z.-L., Momota, Y., Kato, K., Hirata, A., and Shiosaka, S. (1995) Neurosci. Res. 23, 345–351
33. Schechter, I., and Berger, A. (1967) Biochem. Biophys. Res. Commun. 27, 157–162
34. Schechter, I., and Berger, A. (1968) Biochem. Biophys. Res. Commun. 32, 898–902
35. Uchino, T., Sakurai, Y., Nishigai, M., Kakahashi, T., Arakawa, H., Ikai, A., and Takahashi, K. (1993) J. Biol. Chem. 268, 527–533
36. Chen, Z.-L., Momota, Y., Kato, K., Taniguchi, M., Inoue, N., Shiosaka, S., and Yoshida, S. (1997) J. Histochem. Cytochem. 46, 313–320
37. Sanes, J. R. (1983) Trends Neurosci. 15, 541–544
38. Pierschbacher, M. D., Hayman, E. G., and Ruoslahti, E. (1981) Cell 26, 259–267