Role of Loop Structures of Neuropsin in the Activity of Serine Protease and Regulated Secretion*

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Neuropsin involved in neural plasticity in adult mouse brain is a member of the S1 (clan SA) family of serine proteases and forms characteristic surface loops surrounding the substrate-binding site (Kishi, T., Kato, M., Shimizu, T., Kato, K., Matsumoto, K., Yoshida, S., Shiosaka, S., and Hakoshima, T. (1999) J. Biol. Chem. 274, 4220–4224). Little, however, is known about the roles of these loops. Thus, the present study investigated whether surface loop structures of neuropsin were essential for the generation of enzymatic activity and/or secretion of the enzyme via a regulated secretory pathway. The loops include those stabilized by six disulfide bonds or a loop C (Gly69–Glu80) and an N-glycosylated kallikrein loop (His91–Ile103) not containing a site linked by a disulfide bond. First, among the six disulfide bonds, only SS1 in loop E (Gly142–Leu155) and SS6 in loop G (Ser185–Gly197) were necessary for the catalytic efficiency of neuropsin. Second, disruptions of loop C and the N-linked oligosaccharide chain on the kallikrein loop affected the catalytic efficiency and P2 specificity, respectively. Alternatively, disruptions of loop C and the kallikrein loop enhanced the regulated secretion, whereas there was no one disruption that inhibited the secretion, indicating that there was no critical loop required for the regulated secretion among loops surrounding the substrate-binding site.

Several serine proteases have been shown to play important roles in synaptic plasticity (1–3). These functions are suggested to be mediated by the activation of specific cell surface receptors and the degradation of extracellular matrix proteins and cell adhesion molecules (4, 5). Neuropsin is a secretory serine protease expressed predominantly in pyramidal neurons in the hippocampal subfields CA1–3 (6) and is implicated in activity-dependent plasticity changes in neurons (2, 3, 6, 7). The activity of neuropsin is regulated by a specific inhibitor, serine proteinase inhibitor-3, and one disruption that inhibited the secretion, indicating that there was no critical loop required for the regulated secretion among loops surrounding the substrate-binding site.

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EXPERIMENTAL PROCEDURES

Plasmid Construction—pED1-NP was constructed as follows. A 789-bp NcoI-XhoI fragment of a full-length neuropsin cDNA was amplified based on NP1-pBluescript(II)Ks− (6) by PCR using the forward primer 5′-CCG GAT ATC ACT CAG CAT AAT G-3′ (T7 primer) and the reverse primer 5′-GAG CTC TTT TCT TAA GCC TAT C-3′. The amplified product was digested with NcoI and XhoI and ligated into pED1 (9). The pED1-NP construct was confirmed by sequencing.
reverse primer 5'-GGA CTC GAG TCA GTC CCT GTT GTC CAT TGT CTT-3' (primer-A, containing a stop codon and Xhol site) and introduced into the Neo1-Xhol site of pED1 vector (4896 bp) (a gift from Dr. Mahito Nakashiki, Gene Discovery Research Center, AIST, Ibaragi, Japan), which contains the cytomegalovirus enhancer, chicken β-actin promoter, and SV40 late poly(A) signal (27), to generate pED1-NP.

Point mutations were introduced into a full-size neuropsin cDNA of pED1-NP by oligonucleotide-directed mutagenesis using Mutan- Super Express Kpn according to the manufacturer's protocol (TaKaRa, Siga, Japan). The *numerals* in the clone names indicate the amino acid number counted from the start codon, Met. The following primers were used for the nucleotides changed relative to the neuropsin cDNA sequence are underlined: C78, 5'-GCC CCA CCC TGT GCA ATC CA-3'; C398, 5'-AGG TCA AGC GTC TAT ACC CCA C3-3'; C74S, 5'-AGC CCA CTG CAA AAA ACA G-3'; C108S, 5'-GGA TCA TTC CTA CAA CAA C-3'; C145S, 5'-CCA ATC TGT TCG CCA AAG TTG GCC AGA AG-3'; C152S, 5'-TGG GCC AGA AGT CCA TCA TAT GAC G-3'; C188S, 5'-AGG GCA TGG TCT CTT GTG GCA GCA G-3'; C208S, 5'-TGA CAC GTC CCA GGG TG-3'; C233S, 5'-TCA GAC CCC TCT GGG AAA CCC G-3'; C246S, 5'-ACA CAA TTA TCT CCC GCT ACA TTA CC-3'; N110A, 5'-TCC TTA CTA CCG CAA CAG CAA CCC-3'; D206V, 5'-TGG AGC TGC CAC GTG CC-3'; DS211VA, 5'-TGG AGC TGA CAC GTG CCA GGG GTG QC CAG AGG CCC-3'. Deletion mutants of ΔS87-P94 and N1105-ΔN113-E115 (28) were created by PCR with NsiI-XhoI (15). PCR fragments of 250 bp of NcoI-EcoO65I (forward, T7 primer; reverse, 5'-ATG GTC ACC CAG CAG CAC G-3') and 509 bp of EcoO65I-Xhol (forward, 5'-TAC TTC GTG GCA GGT CAT GAG CAG GAG ATC GAT GTG GC-3'; reverse, primer-A) were inserted into the Neo1-Xhol site of pED1 to generate ΔS87-P94. PCR fragments of 328 bp of NcoI-NspV (forward, T7 primer; reverse, 5'-GTT CGA ATA GCA GTA AGC ATG CAT GAT AGA A-3') and 446 bp of NspV-Xhol (forward, 5'-TCT ATC GAC CAG TCT TGX TGT GAC AAG AGC GAT CAT GAC CAC ATG CAC GAT ATA ATG-3'; reverse, primer-A) were inserted into the Neo1-Xhol site of pED1 to generate N1105-ΔN113-E115. 

*pSIIH* vector encoding human growth hormone was described previously (28).

**Cell Culture and Transfection.—**The Neuro2a cell line (mouse neuroblastoma; Institute for Fermentation, Osaka, Japan) was grown in Eagle's medium (Nissui, Tokyo, Japan) supplemented with 1% nonessential amino acids (Invitrogen) and 10% fetal bovine serum at 37 °C in a 5% CO₂ incubator. Neuro2a cells were plated at a density of 1.0 × 10⁶ cells/35-mm dish (1.2 × 10⁶ cells/35-mm dish) in Dulbecco's modified Eagle's medium (Nissui, Tokyo, Japan) supplemented with 1% nones-

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**Quantification of Mutants and Wild-type Neuropsin in Conditioned Media.—**Media and cell lysates were subjected to SDS-PAGE using 10% acrylamide gel. The proteins were transferred to a polyvinylidene di-

The band density stained using colloidal properties of Coomassie G-250 with F12mAb (7), the beads were subjected to reducing SDS-PAGE, and the band intensity was determined based on the stained intensity using one-dimensional gel image analysis software (Quantity One software, PDI, Toyobo Co., Ltd., Osaka, Japan) (8). Reaction with neuropsin (Escherichia coli, purified from a baculovirus expression system (29), was used as a control of the amount.

For comparison of the activity of 11pAb to bind mutants and wild-type neuropsin, conditioned medium derived from each transfectant was immunoprecipitated with Affi-Gel Hx beads (Bio-Rad) conjugated with F12mAb (7), the beads were subjected to reducing SDS-PAGE, and the band density was determined using coiled properties of Coomassie G-250.
Preparation of Active Neuropsin and Assay of Amidolytic Activity—The amidolytic activity of neuropsin was determined basically as described previously (29). Briefly, conditioned media of Neuro2a cells transfected with mutants and wild-type neuropsin were treated with lysyl endopeptidase (EC 3.4.21.50) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) were mixed with 50 nM mutant and wild-type neuropsin in a multiwell plate reader (Cytofluor II, PerSeptive Biosystems, Tokyo, Japan). The reaction proceeded at 25 °C for 0–60 min at 3-min intervals in 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml borine serum albumin, and 0.02% NaN₃ at 25 °C. Representative values for three independent Neuro2a cell transfection experiments were given as averages of triplicate assays.

| TABLE I

Enzymatic activity of mutants and wild type of neuropsin in media of transfected neuro2a cells

Fluorogenic substrate was hydrolyzed in 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml bovine serum albumin, and 0.02% NaN₃ at 25 °C. Representative values for three independent Neuro2a cell transfection experiments were given as averages of triplicate assays.

|        | kₐᵡ | kᵡ | kₐᵡ/Kᵡ |
|--------|------|-----|---------|
|        | s⁻¹  | µM  | M⁻¹s⁻¹  |
| Boc-Val-Pro-Arg-MCA | | | |
| Neuropsin (Baculo)* | 68.8 | 245 | 2.81 × 10⁵ |
| Wild type | 100 | 282 | 3.55 × 10⁵ |
| D206V | 0.141 | 81.9 | 1.72 × 10⁵ |
| DS211VA | 0.0387 | 86.3 | 4.48 × 10⁵ |
| Δ(S87-P94) | 15.8 | 966 | 1.64 × 10⁴ |
| N110S · Δ(N113-E115) | 51.6 | 97.1 | 5.32 × 10⁵ |
| N110A | 45.0 | 152 | 2.96 × 10⁵ |
| C75 | 47.0 | 190 | 2.48 × 10⁵ |
| C108S | 78.3 | 158 | 4.95 × 10⁵ |
| C398 (SS1) | 11.6 | 703 | 1.65 × 10⁴ |
| C145S (SS3) | 50.8 | 110 | 4.60 × 10⁴ |
| C208S (SS6) | 0.513 | 102 | 5.03 × 10⁵ |
| C233S (SS6) | 0.424 | 212 | 2.00 × 10⁵ |
| C246S (SS3) | 28.8 | 119 | 2.43 × 10⁵ |
| Pro-Phe-Arg-MCA | | | |
| Wild type | 193 | 8360 | 2.31 × 10⁴ |
| N110S · Δ(N113-E115) | 49.9 | 1270 | 3.92 × 10⁴ |
| N110A | 18.3 | 410 | 4.47 × 10⁴ |
| Boc-Phe-Ser-Arg-MCA | | | |
| Wild type | 34.8 | 216 | 1.61 × 10⁵ |
| C145S (SS3) | 38.1 | 209 | 1.82 × 10⁵ |
| C246S (SS3) | 31.4 | 238 | 1.32 × 10⁵ |
| N110S · Δ(N113-E115) | 35.7 | 215 | 1.66 × 10⁵ |
| N110A | 24.5 | 180 | 1.36 × 10⁵ |
| Boc-Asp(benzyloxy)-Pro-Arg-MCA | | | |
| Wild type | 31.3 | 316 | 9.92 × 10⁴ |
| C145S (SS3) | 33.1 | 309 | 1.07 × 10⁵ |
| C246S (SS3) | 22.7 | 287 | 7.90 × 10⁴ |

*Neuropsin prepared from baculovirus expression system (28).
Enzymatic Activity and Regulated Secretion of Neuropsin

RESULTS

To investigate the role of the surface loop structure of neuropsin in enzymatic activity and secretion, site-directed mutagenesis was employed in loop C (Gly<sup>69</sup>-Glu<sup>80</sup>), the N-glycosylated kallikrein loop (His<sup>21</sup>-Ile<sup>33</sup>), and six disulfide bonds (Fig. 1B). Neuro2a cells transiently transfected with mutant and wild-type neuropsin cDNA were cultured for 36 h. Disruption of the disulfide bonds SS2, SS4, and SS5 interrupted the secretion and caused the enzymes to distribute in the endoplasmic reticulum but not the Golgi complex (data not shown). Since the results show quality control of the enzymes, they were uninformative with regard to the enzymatic activity and secretion.

Comparison of Enzymatic Activity between Mutant and Wild-type Neuropsin—Twelve mutants and the wild-type neuropsin had little amidolytic activity without treatment by lysyl endopeptidase (data not shown) (29). Table I shows the enzymatic activities of the mutants and wild type detected after treatment with lysyl endopeptidase.

First, the enzymatic activity was examined with Boc-Val-Pro-Arg-MCA. Mutations in Asp<sup>180</sup> (D206V; S1-specific pocket) and Ser<sup>169</sup> (DS211VA; catalytic triad) lacking a protease active pocket resulted in levels of activity ∼200- and ∼800-fold less than the wild type as measured by $k_{cat}/K_m$, respectively (Table I, lines 3 and 4). Alternatively, C208S (SS6) and C233S (SS6), had 70–200-fold less activity than the wild type (Table I, lines 12 and 13), the $k_{cat}$ values being ∼200-fold less than and the $K_m$ values almost the same as the wild-type values. Disruptions of loop C ($\Delta$(S87-P94)) and of a disulfide bond SS1 (C39S) caused hydration of the substrate to occur 22 times more slowly than for the wild-type (Table I, lines 5 and 10). Both loop C ($\Delta$(S87-P94)) and C39S (SS1) showed a decrease in $k_{cat}$ values and increase in $K_m$ values, indicating that loop C (Gly<sup>69</sup>-Glu<sup>80</sup>) and SS1 were necessary for catalytic efficiency. The remaining mutants showed little difference in activity on Boc-Val-Pro-Arg-MCA, relative to the wild-type.

The three-dimensional view has revealed that the kallikrein loop of neuropsin forms a narrow P2 pocket (9). To determine the effect of N-glycosylation of the kallikrein loop on the P2 specificity of neuropsin experimentally, the activities of N110A and N110S-A(N113-E115) were examined with Pro-Phe-Arg-MCA. Almost the same catalytic activity was found as that of the wild type in HK medium (data not shown) (29). Table I shows the enzymatic activities of the mutants and wild type in HK and LK media and 1/4 volumes of cell lysates were run on SDS-PAGE gel and Western blotted with 11pAb.

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to that in low K⁺ medium (Fig. 2A). At the same time, we revealed that the release of transfected neuropsin was significantly stimulated by high K⁺ medium 3.4-fold, and the stimulated secretion was dependent on the presence of calcium (Fig. 2B). Furthermore, immunofluorescence cytochemistry showed that neuropsin distributed as punctate structures in the cytoplasm and neurites of PC12 cells co-localized with chromogranin A (Fig. 4A, arrows). It has been reported that chromogranin A is a major component of large dense core vesicles and plays a key role in regulated secretory granule biogenesis in PC12 cells (34). Thus, the present results are the first evidence that neuropsin was secreted in a regulated manner as well as hGH.

Comparison of the Regulated Secretion of Mutant and Wild-type Neuropsin—To elucidate the roles of each loop of neuropsin in the regulated secretion, the same release assays as employed for the wild type were performed in PC12 cells transfected with mutant cDNAs (Fig. 3). There was no one disruption that inhibited high K⁺-evoked release. Alternatively, high K⁺-evoked releases of three mutants involving loop C (Δ(S87-P94)) and loop D containing an N-linked oligosaccharide chain (N110A and N110S-Δ(N113-E115)) were enhanced to 1.6-, 1.7-, and 2.0-fold relative to that of the wild-type (Fig. 3, stars). All mutant proteins, except those with disrupted disulfide bonds SS2, SS4, and SS5, co-localized with chromogranin A in cytoplasm and neurites like the wild-type, suggesting that no mutants were excluded from the regulated secretory pathway (Fig. 4, B–E). We concluded that there were no surface loops essential for targeting to the regulated secretory pathway among loops disrupted in the present study.

DISCUSSION

Effects of Mutations on Enzymatic Activity of Neuropsin

The present study provides experimental evidence that characteristic surface loops of neuropsin control the specificity of enzymatic activity.

Mutations Affecting the P1 Specificity of Enzyme—Asp¹⁸⁹ in the S1-specific pocket (D206V) and Ser¹⁹⁵ in the catalytic triad (DS211VA) were necessary for the catalytic efficiency of neuropsin (Table I) as well as trypsin (13, 38), and disruption of the disulfide bond SS6 resulted in a remarkable reduction in the enzymatic activity of neuropsin. The crystal structure of neuropsin shows that SS6 provides stability in loop G (Ser¹⁸⁵–Gly¹⁹⁷) (Fig. 1A) (9). And, it was elucidated experimentally that disruption of SS6 induced displacement of loop G and led to a change in the position of Ser¹⁸⁵, part of the catalytic triad, resulting in a decrease of enzymatic activity. Since all S1 (clan SA) serine proteases possess SS6, it was proposed that the role of SS6 was common to all of the members.

Mutations Affecting Association of Enzyme with the C-terminal Side of Substrate—Known structures of family S1 (clan SA) serine proteases indicate that a loop structure similar to loop E (Gly¹⁴²–Leu¹⁵⁵) of neuropsin, which is stabilized by the disulfide bond SS1 (Fig. 1A) (9), is in contact with the substrate on the C-terminal side (13). In addition, loop C (Gly⁹⁰–Glu⁹⁵) of neuropsin is positioned close to loop E (9, 18, 19). The effect of loops C and E on catalytic efficiency indicates that interaction of the enzyme with the extended substrate generally helps to maintain catalytic efficiency. On the other hand, a previous report has shown that the loop structure of trypsin similar to loop C of neuropsin contains a calcium binding site and is involved in autolysis but not enzymatic activity (18, 39). Thus, the possibility remains that the effect of the loop C of neuropsin on catalytic efficiency is specific for neuropsin.

Mutations Affecting the P2 Specificity of Enzyme—The three-dimensional view has indicated that the kallikrein loop structure (His⁸⁴–Ile¹⁰³) of neuropsin affects the S2 site (Fig. 1A) (9). In the present study, it was indeed shown that N-linked oligosaccharides on the kallikrein loop affected the size of the P2 pocket. Additionally, N110A was more effective against P2 specificity than was N110A-Δ(N113-E115). The crystal structure indicates that Glu⁹⁷ is projected from the surface to the S1 pocket. Thus, deletion of only N-linked oligosaccharides might also affect the projection of Glu⁹⁷, resulting in an increase of nonproductive binding. Alternatively, since the kallikrein loop of neuropsin differs radically from other members of the kallikrein family as already mentioned (9, 19), the present effect of N-linked oligosaccharides on P2 specificity might be only specific for neuropsin.

Effects of Mutations on Regulated Secretion of Neuropsin

The present results indicated that neuropsin was secreted in a regulated manner. A previous study suggested that a protease cleavage event at a dibasic site of an aspartyl protease, prorenin, by proprotein convertases is implicated in the regulated secretion of renin (40). Alternatively, in family S1 (clan SA) serine proteases, previous studies have indicated that the
loops, loop C and the N-linked oligosaccharide chain on the kallikrein loop, however, enhanced the secretion, suggesting the involvement of these sites in the secretion.

Concerning several prohormone and propropptide convertases, the amphipathic helical segment is essential for regulated secretion, disruptions causing a severe reduction in the secretion (41–46). Regarding neuropsin, the only candidate for the amphipathic helical loop among the surface loops that surround the substrate-binding site is loop E (9). In the present study, disruption of loop E, however, caused no reduction in regulated secretion, whereas the effect of the disruption on catalytic efficiency confirms perturbation of the loop E structure. On the other hand, the present study indicated that disruption of loop C caused an enhancement of regulated secretion, whereas no experiments have shown, so far, disruptions to any molecules that cause an increase in regulated secretion (20, 41, 42, 45, 46). Loop C consists of hydrophilic amino acids and is projected to the surface (9) and the functional significance of the increase could not be elucidated in the present study. Thus, it is first necessary to determine whether the increase is common to other S1 (clan SA) serine proteases.

In addition, site-directed removal of the N-glycosylation site of neuropsin also enhanced the regulated secretion. The experiments on enzymatic activity confirmed that N-linked oligosaccharides provide structural rigidity to the kallikrein loop, which determines the size of the P2 pocket. Thus, changes in the kallikrein loop structure caused by the removal of N-linked oligosaccharides might also induce an enhancement of secretion. On the other hand, previous reports have shown that appropriate glycosylation of a number of proteins is important for proper expression and function (i.e. stability, folding in the endoplasmic reticulum, trafficking to the Golgi complex and plasma membrane, and catalytic activity) (47–55). It remains, therefore, possible that variability among sugar structures in the kallikrein loop affects the regulated secretion directly.

Finally, the present results provide new information on the structure-function of family S1 (clan SA) serine proteases.

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