THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 277, No. 17, Issue of April 26, pp. 14724–14730, 2002
Printed in U.S.A.

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

Received for publication, November 8, 2001, and in revised form, February 15, 2002
Published, JBC Papers in Press, February 19, 2002, DOI 10.1074/jbc.M110725200

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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 (Gly^{69}–Glu^{80}) and an *N*-glycosylated kallikrein loop (His^{91}–Ile^{103}) not containing a site linked by a disulfide bond. First, among the six disulfide bonds, only SS1 in loop E (Gly^{142}–Leu^{155}) and SS6 in loop G (Ser^{185}–Gly^{197}) 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, in adult mouse brain (8).

The crystal structure of neuropsin has a serine protease fold that exhibits chimeric features of trypsin and nerve growth factor-γ, a member of the kallikrein family (Fig. 1*A*) (9), both of which are a part of the S1 family (clan SA) of serine proteases (10). All S1 serine proteases possess two β-barrels with a catalytic His^{57} (chymotrypsin position number), Asp^{102}, and Ser^{195} located at the interface of the two domains (10). These proteases, however, show diversity in the structures of surface loops surrounding the substrate-binding site, and it has been proposed that this diversity controls the specificity of enzymatic activity (9, 11–14). Four to six disulfide bonds form, which probably provide a degree of structural rigidity to the loop structures (9, 15, 16). Neuropsin possesses six disulfide bonds, the same as trypsin. Furthermore, neuropsin forms a loop C (Gly^{69}–Glu^{80}) and an *N*-glycosylated loop D (17), the "kallikrein loop" (His^{91}–Ile^{103}), not containing a site linked by a disulfide bond. The loop C of neuropsin has been superimposed on that of trypsin and nerve growth factor-γ (9, 18, 19). On the other hand, the kallikrein loop is present in all members of the kallikrein family but not trypsin. However, the kallikrein loop of neuropsin differs radically from that of nerve growth factor-γ and of kallikrein (9, 19). While the loop of most members of the kallikrein family is cleaved into highly mobile nicked chains, that of neuropsin is packed without any nicked sites. This three-dimensional view of neuropsin provides the opportunity to examine the correlation between structure and substrate specificity (9), and it is necessary to evaluate the correlation experimentally.

Most family S1 (clan SA) serine proteases are synthesized as precursors and then enter the secretory pathway (10). It has been indicated that some are sorted to a regulated secretory pathway (20–23). For example, the secretion of trypsinogen, elastase, and cathepsin G is clearly regulated (20, 21), whereas it is debatable whether kallikrein is secreted in a regulated manner (24, 25). However, it is not known which domain of these proteases is involved in regulated secretion. On the other hand, there is physiological evidence that neuropsin is involved in activity-dependent synaptic plasticity (2, 3, 6, 7). However, it is still not clear whether neuropsin is secreted in response to stimuli. It is, thus, necessary to determine whether a secretagogue causes exocytotic release of neuropsin and, if so, which domain of neuropsin is required for regulated secretion.

In the present study, site-directed removal involving six disulfide bonds, a loop C, and an *N*-glycosylated kallikrein loop of neuropsin was carried out, and the effects of these mutations on the enzymatic activity and the regulated secretion were investigated.

EXPERIMENTAL PROCEDURES

Plasmid Construction—pED1-NP was constructed as follows. A 789-bp *Nco*I-*Xho*I fragment of a full-length neuropsin cDNA was amplified based on NP1-pBluescript(II)KS^+ (6) by PCR using the forward primer 5′-CGG GAT ATC ACT CAG CAT AAT G-3′ (T7 primer) and

* This work was supported in part by grants from the Ministry of Education, Culture, Sports, Science, and Technology of Japan and the Japan Society for the Promotion of Science in Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by a research fellowship for young scientists from the Japan Society for the Promotion of Science.

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reverse primer 5′-GGA *CTC GAG* TCA GTC CCT GTT GTC CAT TGT CTT-3′ (primer-A, containing a stop codon and *Xho*I site) and introduced into the *Nco*I-*Xho*I site of pED1 vector (4896 bp) (a gift from Dr. Mahito Nakanishi, Gene Discovery Research Center, AIST, Ibaragi, Japan), which contains the cytomegalovirus enhancer, chicken β-actin promoter (26), 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 K_m 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, and the nucleotides changed relative to the neuropsin cDNA sequence are underlined: C7S, 5′-CCC CCA CCC TCT GCA ATC CA-3′; C39S, 5′-AGG TCG AGA GTC TAT ACC CCA C-3′; C74S, 5′-AGC CCA CTC CAA AAA ACA G-3′; C108S, 5′-GCA TCC TTC CTA CAA CAA C-3′; C145S, 5′-CCA ATC TGT CTC CCA AAG TTG GCC AGA AG-3′; C152S, 5′-TTG GCC AGA AGT *C*CA TCA TAT CAG G-3′; C198S, 5′-AGG GCA TGG TCT CTG CTG 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 CCA AAA TCT CCC GCT ACA CTA CC-3′; N110A, 5′-TCC TTG CTA CGC CAA CAG CAA CCC-3′; D206V, 5′-TGG AGC TGT CAC GTG CC-3′; DS211VA, 5′-TGG AGC TGA CAC GTG CCA GGG TGT CGC AGG AGG CCC-3′. Deletion mutants of Δ(S87-P94) and N110S·Δ(N113-E115) were created by PCR with NP1-pBluescript(I-I)KS$^+$ (6). PCR fragments of 250 bp of *Nco*I-EcoO65I (forward, T7 primer; reverse, 5′-ATG *G*TC ACC CAG ACG CAC G-3′) and 509 bp of *Eco*O65I-*Xho*I (forward, 5′-TAC TCC GTG CGT CTG GGT GA*C* CAT GAG CAG GAG ATC CAG GTG GC-3′; reverse, primer-A) were inserted into the *Nco*I-*Xho*I site of pED1 to generate Δ(S87-P94). PCR fragments of 328 bp of *Nco*I-*Nsp*V (forward, T7 primer; reverse, 5′-GTT CGA ATA GCA AGG ATG CTG GAT AGA-3′) and 446 bp of *Nsp*V-*Xho*I (forward, 5′-TCT ATC CAG CAT CCT TGC TAT TCG AAC AGC GAT CAC AGT CAC GAT ATA ATG-3′; reverse, primer-A) were inserted into the *Nco*I-*Xho*I site of pED1 to generate N110S·Δ(N113-E115). pSIhGH 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% CO_2 incubator. Neuro2a cells were plated at a density of $1.0 \times 10^4/cm^2$ (1.0×10^5 cells/35-mm dish) in Eagle's medium, 10% FCS, and 1% nonessential amino acids on a coverslip (Matsunami, Osaka, Japan) coated with poly-L-lysine (4 μg/cm^2; Sigma). After 18 h, cells were transfected with 1 μg of pED1-NP and the mutants in OPTI-MEM plus 1% nonessential amino acids by LipofectAMINE PLUS (Invitrogen). After 3 h, the medium was replaced with fresh OPTI-MEM plus 1% nonessential amino acids containing N-2 supplement (differentiation) (Invitrogen). Conditioned medium (2 ml/35-mm dish) was recovered after 36 h of culture for the enzyme assay. The PC12 cell line (a gift from Dr. Yasuhisa Hukui, University of Tokyo, Japan) was grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 5% fetal bovine serum and 5% heat-inactivated horse serum at 37 °C in a 10% CO_2 incubator. PC12 cells were plated at a density of 1.2×10^5 cells/cm^2 (1.2×10^6 cells/35-mm dish) in Dulbecco's modified Eagle's medium, 5% fetal bovine serum, and 5% horse serum on a dish coated with polyethyleneimine (Sigma). After 18 h, cells were co-transfected with 1.5 μg of pSIhGH and 1.5 μg of pED1-neuropsin by LipofectAMINE 2000 (Invitrogen). After 48 h, assays of hGH and neuropsin release were performed.

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 difluoride membrane (Bio-Rad). The membrane was reacted with rabbit anti-neuropsin polyclonal antibody (11pAb) (8) and then goat anti-rabbit IgG conjugated with alkaline phosphatase (Bio-Rad) in 5% skim milk in 0.1 M Tris-HCl, pH 7.5, 0.15 M NaCl, and 0.1% Tween-20. The secondary antibody was detected by enhanced chemiluminescence (Immun-Star Substrate, Bio-Rad), followed by exposure to x-ray film. The amount of mutant and wild-type protein was determined based on the band densities using one-dimensional gel image analysis software (Quantity One software, PDI, Toyobo Co., Ltd., Osaka, Japan) (8). Recombinant neuropsin (Baculo), 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 Hz beads (Bio-Rad) conjugated with F12mAb (7), the beads were subjected to reducing SDS-PAGE, and the band density stained using colloidal properties of Coomassie G-250

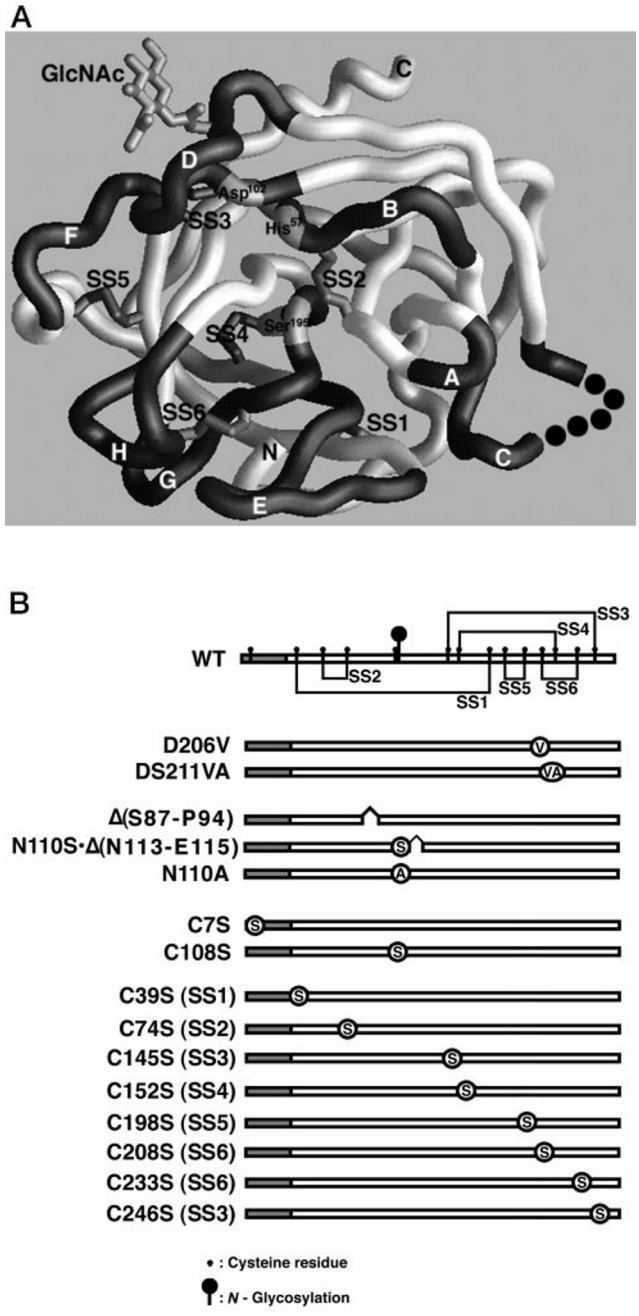

FIG. 1. **Structure of mutant and wild-type neuropsin.** *A*, overall structure of neuropsin (Protein Data Bank code 1NPM) (9). Note that the *N*-glycosylated loop D is the "kallikrein loop" preserved among the kallikrein family. The catalytic triad consists of His^{57} (chymotrypsin number), Asp^{102}, and Ser^{195}; loop structures are labeled *A–H* (*white characters*); N- and C-termini are labeled *N* and *C* (*black characters*); and disulfide bonds are marked *SS1–SS6*. *B*, schematic representation of neuropsin and the established mutations. At the *top*, the *N*-glycosylation site (*large lollipop symbol*), two free cysteines (*small closed circles*), and six disulfide bonds (*SS1–SS6*) are indicated (*WT*, wild-type neuropsin). The ER signal sequence is marked with *hatched boxes*. The *numerals* in the clone names indicate the amino acid number counted from the start codon, Met. To obtain mutants disrupted at the protease active pocket, Ala or Val was substituted for Asp^{189} at the S1-specific pocket (D206V) and for Asp^{194} and Ser^{195} in the catalytic triad (DS211VA). A deletion of Ser^{72}–Pro^{79} at loop C was performed (Δ(S87-P94)). In the *N*-glycosylated kallikrein loop, Asn^{95}, the glycosylation site was changed to Ala (N110A), and the three amino acids Asn^{95}–Glu^{97} were deleted in addition to substitution of Asn^{95} with Ser (N110S·Δ(N113-E115)). Ser was substituted for free cysteine residues (C7S and C108S) and for Cys^{22} (SS1), Cys^{58} (SS2), Cys^{128} (SS3), Cys^{136} (SS4), Cys^{182} (SS5), Cys^{191} (SS6), Cys^{220} (SS6), and Cys^{232} (SS3) to disrupt the disulfide bonds (C39S, C74S, C145S, C152S, C198S, C208S, C233S, and C246S).

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 borine serum albumin, and 0.02% NaN_3 at 25 °C. Representative values for three independent Neuro2a cell transfection experiments were given as averages of triplicate assays.

	k_{cat}	K_m	k_{cat}/K_m
	s^{-1}	μM	$_M{}^{-1}s^{-1}$
Boc-Val-Pro-Arg-MCA			
Neuropsin (Baculo)[a]	68.8	245	2.81×10^5
Wild type	100	282	3.55×10^5
D206V	0.141	81.9	1.72×10^3
DS211VA	0.0387	86.3	4.48×10^2
Δ(S87-P94)	15.8	966	1.64×10^4
N110S · Δ(N113-E115)	51.6	97.1	5.32×10^5
N110A	45.0	152	2.96×10^5
C7S	47.0	190	2.48×10^5
C108S	78.3	158	4.95×10^5
C39S (SS1)	11.6	703	1.65×10^4
C145S (SS3)	50.8	110	4.60×10^5
C208S (SS6)	0.513	102	5.03×10^3
C233S (SS6)	0.424	212	2.00×10^3
C246S (SS3)	28.8	119	2.43×10^5
Pro-Phe-Arg-MCA			
Wild type	193	8360	2.31×10^4
N110S · Δ(N113-E115)	49.9	1270	3.92×10^4
N110A	18.3	410	4.47×10^4
Boc-Phe-Ser-Arg-MCA			
Wild type	34.8	216	1.61×10^5
C145S (SS3)	38.1	209	1.82×10^5
C246S (SS3)	31.4	238	1.32×10^5
N110S · Δ(N113-E115)	35.7	215	1.66×10^5
N110A	24.5	180	1.36×10^5
Boc-Asp(benzyloxy)-Pro-Arg-MCA			
Wild type	31.3	316	9.92×10^4
C145S (SS3)	33.1	309	1.07×10^5
C246S (SS3)	22.7	287	7.90×10^4

[a] Neuropsin prepared from baculovirus expression system (28).

(Gelcode blue; Pierce) was compared with the band density obtained by Western blotting with 11pAb (8). There was no difference in the band density of mutants and wild-type neuropsin by Western blotting with 11pAb per μg of protein contents (data not shown).

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) conjugated with Sepharose 4B (Amersham Biosciences) at 37 °C for 15 min, and then the amount of mutant and wild-type neuropsin in the medium was determined based on the band density, following reducing SDS-PAGE and immunoblotting. To determine the amidolytic activity, 12.5–200 mM Boc-Val-Pro-Arg-4-methylcoumaryl-7-amide (MCA), Pro-Phe-Arg-MCA, Boc-Phe-Ser-Arg-MCA, and Boc-Asp (benzyloxy)-Pro-Arg-MCA (Peptide Institute, Inc., Osaka, Japan) were mixed with 50 nM mutant and wild-type neuropsin in 96-well plates (Corning Costar, 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 bovine serum albumin, and 0.02% NaN_3 and was monitored with a multiwell plate reader (Cytofluor II, PerSeptive Biosystems, Tokyo, Japan). The kinetic parameters K_m and k_{cat} were obtained by linear regression analysis of the Lineweaver-Burk plot.

Depolarization-induced Release—After 48 h of gene transfection, PC12 cells were washed four times with a low K^+ solution (140 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-NaOH, pH 7.4). They were incubated for 2 min with the low K^+ solution and then for 2 min with a high K^+ solution (140 mM NaCl, 59.7 mM KCl, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 11 mM glucose, and 15 mM HEPES-NaOH, pH 7.4), and each medium was recovered. Cell lysates were homogenized in 10 mM HEPES-NaOH, pH 7.4, and 0.2 mM EDTA using a sonicator (Ultrasonic Homogenizer, VP-5S, TAITEC, Co., Ltd., Saitama, Japan) and centrifuged at 17,500 × *g* for 5 min, and then the supernatant was collected. Contents of human growth hormone (hGH) were determined using an hGH enzyme-linked immunosorbent assay kit (Roche Molecular Biochemicals, Mannheim, Germany) (28). Mutants and wild-type neuropsin were concentrated by acid precipitation with incubation in 6% trichloroacetic acid, 0.0125% deoxycholic acid, and 100 μg/ml gelatin on ice for 1.5 h and centrifugation at 17,500 × *g* for 45 min, and then their content was determined by SDS-PAGE followed by Western blotting with 11pAb. Secretion was expressed as a percentage of total cellular hGH, wild-type neuropsin, and the mutants.

Immunofluorescence—The following antibodies were used in immunofluorescence: rabbit anti-neuropsin polyclonal antibody (11pAb, 20 μg/ml), rat anti-neuropsin monoclonal antibody (B5mAb, 20 μg/ml, Medial and Biological Laboratories Co., Ltd., Nagoya, Japan) (7), mouse anti-Grp78 monoclonal antibody (diluted 1:200; StressGen Biotechnologies Corp., Victoria, BC, Canada) (30), rabbit antiserum against α-mannosidase II, a Golgi enzyme, (1:1,000, a gift from Dr. Kelly Moremen, University of Georgia, Athens, GA) (31), and rabbit anti-chromogranin A polyclonal antibody (1:500, a gift from Dr. Seung Hyun Yoo, KAIST, Korea) (32) as primary antibodies and goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (1:600, BIOSOURCE International, Camarillo, CA), goat anti-rat IgG conjugated with rhodamine (1:100; BIOSOURCE International), and goat anti-mouse IgG conjugated with fluorescein isothiocyanate (1:600; BIOSOURCE International) as secondary antibodies.

Cells were fixed with 4% paraformaldehyde in Dulbecco's phosphate-buffered saline containing 0.7 mM $CaCl_2$ and 0.5 mM $MgCl_2$, at 4 °C for 1 h and permeabilized with 0.2% Triton X-100, 20 mM glycine, and phosphate-buffered saline at 37 °C for 10 min. After a wash with 0.15 M NaCl, 20 mM boric acid, and 5 mM sodium tetraborate decahydrate, pH 8.0 (BBS), cells were incubated with BBS containing 3% bovine serum albumin at room temperature for 30 min and then with primary antibodies in BBS containing 3% bovine serum albumin at 4 °C overnight. After a wash with BBS, secondary antibodies in BBS containing 3% bovine serum albumin were applied at 4 °C overnight. After a wash with BBS, coverslips were mounted in glycerol-containing Mowiol 4–88 (Calbiochem-Novabiochem) and 1,4-diazobicyclo-(2,2,2)-octane (Sigma) (33) and were observed with a Laser scan microscope LSM510 invert (Carl Zeiss, Tokyo, Japan). Double-labeled immunofluorescence cytochemistry with anti-Grp78, anti-α-mannosidase II, and anti-chromogranin A antibodies was used to observe the localization of mutant and wild-type neuropsin on the endoplasmic reticulum (30), Golgi complex (31), and large dense core vesicles (34), respectively.

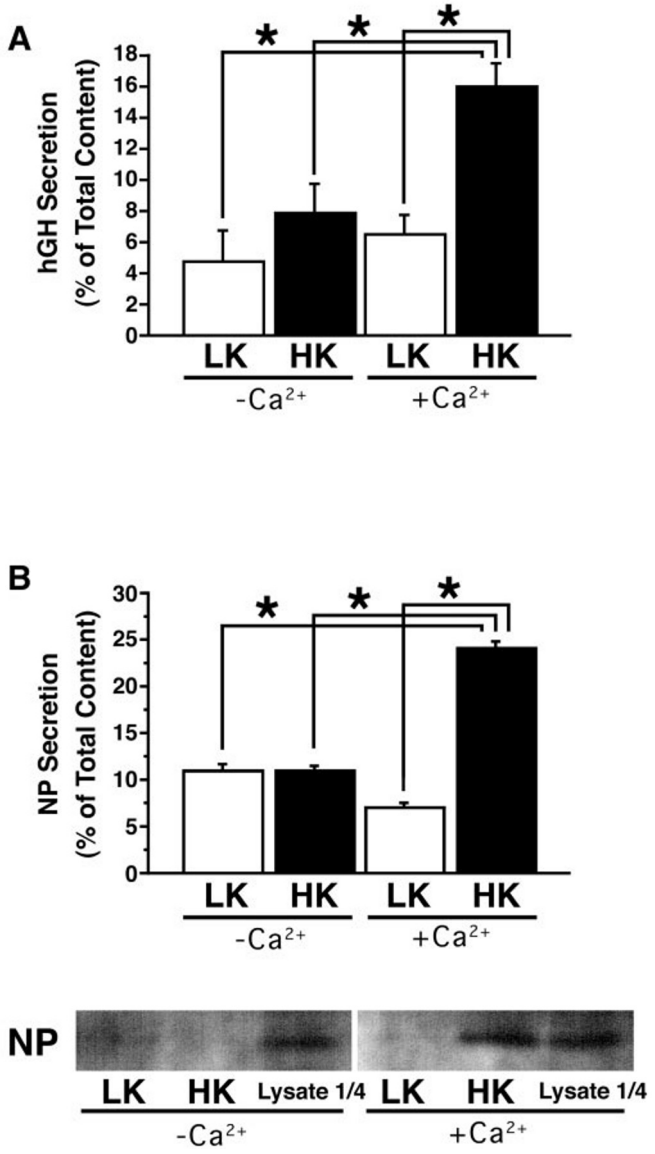

FIG. 2. **Ca^{2+} and depolarization-dependent release of neuropsin from neuropsin-transfected PC12 cells.** PC12 cells were co-transfected with pSIhGH and pED1-NP. Forty-eight hours after transfection, hGH and neuropsin releases were assayed in either low K^+ (*LK*; 4.7 mM KCl) or high K^+ solution (*HK*; 59.7 mM KCl), in the presence or absence of 2.5 mM Ca^{2+}. The amounts of released hGH and neuropsin were expressed as a percentage of the cellular content. *Error bars* show the S.E. of three independent experiments. *A*, high K^+-dependent hGH release. *Stars* show statistical significance against $+Ca^{2+}$/HK as determined by one-way analysis of variance followed by a Bonferroni/Dunn test for multiple comparisons ($F(3, 16) = 8.4287$, $p < 0.002$). *B*, high K^+-dependent neuropsin (*NP*) release. *Stars* show statistical significance against $+Ca^{2+}$/HK as determined by one-way analysis of variance followed by a Bonferroni/Dunn test for multiple comparisons ($F(1, 6) = 36.4651$, $p < 0.001$). At the *bottom*, representative bands of neuropsin are shown. Neuropsin in HK and LK media and ¼ volumes of cell lysates were run on SDS-PAGE gel and Western blotted with 11pAb.

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^{69}–Glu^{80}), the *N*-glycosylated kallikrein loop (His^{91}–Ile^{103}), and six disulfide bonds (Fig. 1*B*). 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^{189} (D206V; S1-specific pocket) and Ser^{195} (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 (Δ(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 (Δ(S87-P94)) and C39S (SS1) showed a decrease in k_{cat} values and increase in K_m values, indicating that loop C (Gly^{69}–Glu^{80}) 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·Δ(N113-E115) were examined with Pro-Phe-Arg-MCA. Almost the same catalytic activity was found as that of the wild type in k_{cat}/K_m (Table I, lines 15–17). However, the k_{cat} values were 11- and 4-fold less than that of the wild-type, and K_m values were 20- and 7-fold lower, indicating enhancement of nonproductive binding of phenylalanine to the S2 site of neuropsin.

The crystal structure indicates that Lys^{175} of loop F is projected toward the S3/S4 site (9), and experimentally, a high level of activity of wild-type neuropsin is observed for synthetic tripeptide substrates having hydrophobic and acidic residues at the P3 position (29). Since the disulfide bond SS3 provides structural rigidity to loop F (Fig. 1*A*), we determined the effect of SS3 on the P3 specificity of neuropsin by examining the activity of C145S and C246S (SS3) with Boc-Phe-Ser-Arg-MCA and Boc-Asp (benzyloxy)-Pro-Arg-MCA. There was no effect on the activities (Table I, lines 18–20 and 23–25). It was suggested that the disulfide bond SS3 had no effect on the P3 specificity of neuropsin.

Regulated Secretion of Neuropsin—Next, we examined whether wild-type neuropsin could be secreted in a regulated manner. First, we determined the amount of neuropsin secreted after 15 min of exposure to high K^+ medium in Neuro 2a cells transfected with wild-type neuropsin cDNA transiently. The result was an increase in the amount by 1.2-fold ($p < 0.05$, Student's *t* test) relative to that in low K^+ medium (data not shown). This showed that Neuro2a cells secreted low levels of neuropsin in a regulated manner. However, the possibility remained that regulated secretion of neuropsin occurred in an alternate cell line (35, 36). Therefore, we next investigated the secretory manner of neuropsin in PC12 cells co-transfected with hGH and neuropsin cDNAs transiently. hGH has been well characterized as a regulated secretory protein (37), and the present study also showed that, after 2 min of exposure to high K^+ medium in the presence of calcium, the release of transfected hGH was significantly stimulated 2.5-fold relative

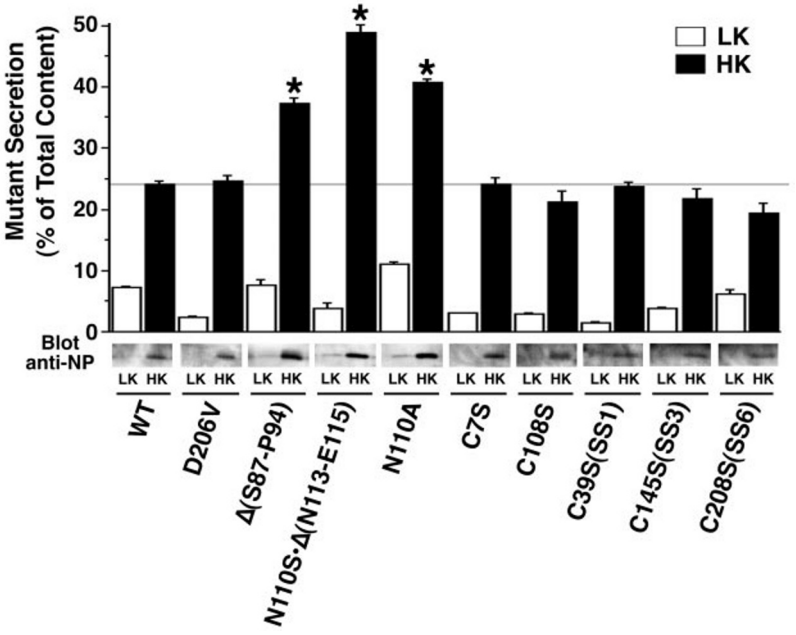

FIG. 3. **Comparison of high K^+-dependent release between mutant and wild-type neuropsin.** PC12 cells were transfected with pED1 mutants or pED1-NP. Forty-eight hours after transfection, releases of mutant and wild-type neuropsin were assayed in either low K^+ or high K^+ solution, in the presence of Ca^{2+}. Percentages of release of wild type are as in Fig. 2*B*. *Error bars* show the S.E. of three independent experiments. *Stars* show statistical difference against percentage release of wild-type as determined by one-way analysis of variance followed by a Bonferroni/Dunn test for multiple comparisons ($F(9, 28) = 75.7756$, $p < 0.0001$). Note that high K^+-dependent releases of Δ(S87-P94), N110A, and N110S·Δ(N113-E115) were more inducible than that of wild type. At the bottom, representative bands of mutant and wild-type neuropsin subjected to SDS-PAGE and Western blotting with 11pAb are shown.

to that in low K^+ medium (Fig. 2*A*). 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. 2*B*). Furthermore, immunofluorescence cytochemistry showed that neuropsin distributed as punctate structures in the cytoplasm and neurites of PC12 cells co-localized with chromogranin A (Fig. 4*A*, *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 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 conclude 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—Asp^{189} in the S1-specific pocket (D206V) and Ser^{195} 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 (Ser^{185}–Gly^{197}) (Fig. 1*A*) (9). And, it was elucidated experimentally that disruption of SS6 induced displacement of loop G and led to a change in the position of Ser^{195}, 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 (Gly^{142}–Leu^{155}) of neuropsin, which is stabilized by the disulfide bond SS1 (Fig. 1*A*) (9), is in contact with the substrate on the C-terminal side (13). In addition, loop C (Gly^{69}–Glu^{80}) 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 (His^{91}–Ile^{103}) of neuropsin affects the S2 site (Fig. 1*A*) (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 Glu^{97} is projected from the surface to the S1 pocket. Thus, deletion of only *N*-linked oligosaccharides might also affect the projection of Glu^{97}, 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 prosegment 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

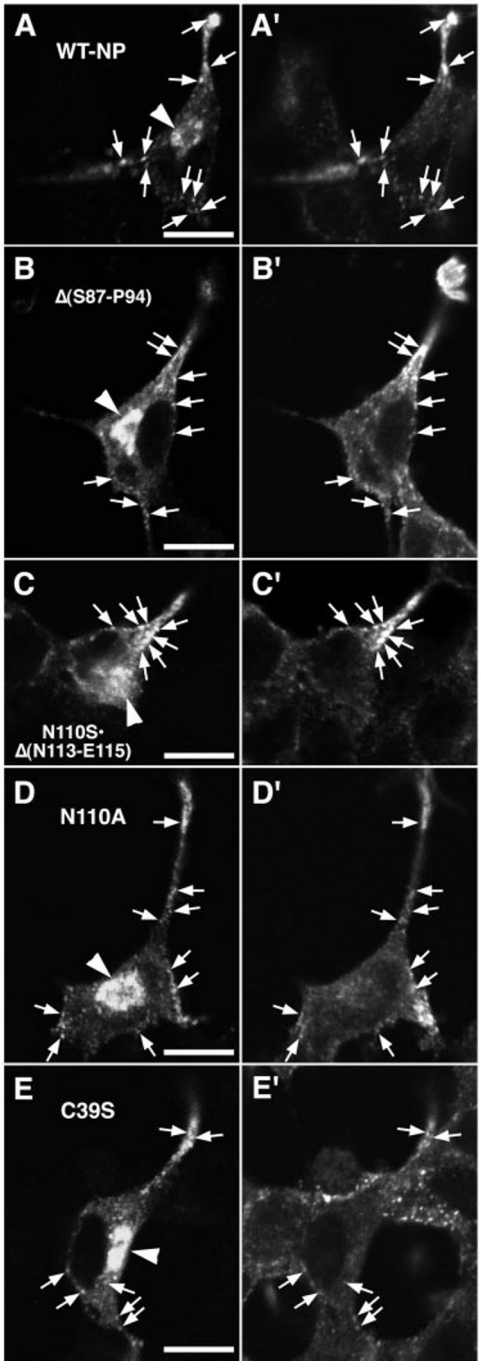

FIG. 4. **Co-localization of mutant and wild-type neuropsin with chromogranin A (CGA) in PC12 cells.** Double-labeled immunofluorescence cytochemistry with anti-neuropsin (B5mAb) (*A–E*) and rabbit anti-chromogranin A (*A′–E′*) antibodies. PC12 cells transfected with wild-type or mutant neuropsin (*AA′*, WT-NP; *BB′*, Δ(S87-P94); *CC′*, N110A; *DD′*, N110S·Δ(N113-E115); *EE′*, C39S). Note that mutants and wild-type neuropsin signals localized as punctate structures in cell bodies and their neurites, where chromogranin A signals co-localized (*arrows*). Neuropsin signals in Golgi complex were determined by double-labeled immunofluorescence as described under "Experimental Procedures" (*arrowheads*). *Bar*, 10 μm.

prosegment of neither trypsinogen nor cathepsin G is essential for sorting to the regulated secretory pathway (20, 23). Therefore, the possibility has remained that a site other than the prosegment is relevant for sorting in the regulated manner. The present study investigated effects of disruptions in loop structures of neuropsin on the regulated secretion and showed that no one critical loop is required for the secretion. Among 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 proprotein convertases, the amphipathic helical segments are 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 been 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.

Acknowledgments—We thank Dr. Mahito Nakanishi for providing pED1 vector, Dr. Yasuhisa Hukui for providing PC12 cells, Masao Shibata for providing anti-neuropsin monoclonal antibodies, Dr. Kelley Moremen for providing rabbit anti-α-mannosidase II antiserum, Dr. Seung Hyun Yoo for providing rabbit anti-chromogranin A antiserum, and Dr. Yasuhiko Horiguchi and Dr. Tamotsu Yoshimori for critical reading of the manuscript.

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