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The structure-function relationship between peptide aldehyde derivatives on initiation of neurite outgrowth in PC12h cells

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We have previously shown that, among many protease inhibitors examined, only a leupeptin analogue, Ac-Leu-Leu-Nle-al (ALLNal), induces neurite outgrowth in PC12h cells. Since this neurite outgrowth is different from that induced by nerve growth factor (NGF) in terms of morphology and persistance, the existence of a specific protease which regulates neurite formation in PC12h cells was expected. A set of 10 ALLNal analogue peptide protease inhibitors was synthesized and examined for their potency in inducing neurite outgrowth in PC12h cells. Substitution of the N-terminal acetyl residue in ALLNal by benzyloxycarbonyl (Z) increased the activity by about 4 times. For Z-Leu-Leu-X-al, neurite outgrowth was induced in the following order: Leu > Phe > Nva > Val = Ile = Nle > Ala > Gly at the X residue. The potency of Z-Leu-Leu-Leu-al (ZLLLal) was 50-fold stronger than that of ALLNal. ZLLLal provides a strong tool for characterizing this new type of protease.

Proteases are postulated to be involved in many cellular events such as proliferation and differentiation. Neuritogenesis is an important step in neuronal differentiation. It is known that neurons of the peripheral and central nervous systems secrete proteases during development [6, 9, 11], and that inhibitor proteins of these proteases released from heart and glial cells [1, 10] induce neurite outgrowth in neuronal cells [8, 10, 14].

Recently we found that only a tripeptide aldehyde protease inhibitor (Ac-Leu-Leu-Nle-al) among many other protease inhibitors examined initiates neurite outgrowth in PC12h cells [13], a simple, homogenous system for studying initial events in neuronal differentiation [2]. This suggests the existence of a highly specific protease which modulates neurite initiation in PC12h cells. To characterize the protease further, we synthesized 10 synthetic protease inhibitors, all variants of ALLNal, and examined their abilities to produce neurite outgrowth in PC12 cells. We found that benzyloxycarbonyl (Z)-Leu-Leu-X-al, where X is a hydrophobic residue, and in particular, Leu, induces neurite outgrowth at low concentrations.

The leupeptin analogue, Ac-Leu-Leu-Nle-al (ALLNal) was from Nacalai Tesque (Kyoto). Bovine skin collagen (collagen type I: 0.5% solution in 1 mM HCl) was purchased from Koken (Tokyo). The variants Z-Leu-Leu-X-al (X = Gly, Ala, Nle, Val, Ile, Nva, Phe, and Leu), Z-Leu-Leu-al, and Z-Leu-Leu-Leu-Leu-al were synthesized according to the method of Ito et al. [4]. Each derivative was confirmed by HPLC, the 2,4-dinitrophenylhydrazine test, and fast atom bombardment mass spectrometry (DX304/DX304: JEOL) with a mass data system (JMA-DAG-5000).

All procedures were the same as described before [13]. In brief, PC12h cells were grown in medium containing 90% DMEM, 5% newborn calf serum, and 5% heat-inactivated horse serum in plastic tissue flasks at 37°C in a 10% CO_2 incubator. For the neurite outgrowth assay, the PC12h cells [5] were plated onto collagen-coated plates with various aldehyde derivatives in serum-free chemically defined N2 medium. At various intervals, cells were fixed and assayed under a phase-contrast microscope by determining the percentage of cells with neuritic processes longer than the diameter of the cell.

We have previously reported that among various protease inhibitors, only a leupeptin analogue, Ac-Leu-Leu-Nle-al (ALLNal), induces neurite outgrowth in PC12h cells on plates coated with purified collagen type I [13]. Morphologically, this neurite outgrowth was different from that induced by nerve growth factor (NGF), and ALLNal has a combinational effect with NGF [12]. NGF induces many neurites from a single cell body, but ALLNal induces the initiation of one or two long neurites from a single cell [13]. Since the neurite-initiating

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activity of ALLNal as well as its protease inhibitor activity is lost on reduction of the functional aldehyde group to an alcohol, ALLNal functions as a neurite initiating factor through its protease inhibitor activity and the existence of a specific protease regulating neurite outgrowth is suggested [13]. Because ALLNal inhibits calcium-dependent neutral protease (CANP or calpain) activity [13], there is a possibility that the candidate protease is CANP. However, other cysteine protease inhibitors (leupeptin, E64c, E64d) have no effect on neurite outgrowth in PC12h cells [13]. There are several reports that inhibitors of thrombin-like proteases and/or plasminogen activator increase neurite outgrowth [3, 8, 10, 14]. However, hirudin (a thrombin inhibitor) and 6-aminocapric acid (urokinase-type plasminogn activator inhibitor) have no effect on neurite outgrowth in PC12h cells [13]. Moreover, the addition of various concentrations of CANP, thrombin, urokinase-type plasminogen activator, or tissue-type plasminogen activator does not alter ALLNal-induced neurite initiation in our assay system (data not shown). The target protease of ALLNal may thus be a new type of protease with a higher reactivity with ALLNal than CANP.

To characterize this protease further, various analogues of ALLNal were synthesized and examined for their effects on neurite formation in PC12h cells. Replacement of the N-terminal acetyl residue with benzyloxycarbonyl (Z) (Z-Leu-Leu-Nle-al: ZLLNal) results in a reduction in the optimal concentration from 1.6 μM to 0.3 μM. Thus it seems likely that the hydrophobic Z group at the N-terminus is more favourable than less hydrophobic acetyl group for interaction with the target protease and for penetration through cytoplasmic membrane if the target protease is intracellular as described below.

TABLE I

EFFECT OF PEPTIDE ALDEHYDE PROTEASE INHIBITORS ON NEURITE OUTGROWTH

Cells were cultured for 48 h with the additive indicated. Figures are mean ± S.E.M. for 3 duplicates. A, acetyl; Z, benzyloxycarbonyl.

Protease inhibitors	Optimal concentrations (μM)	% cells with neurites
Ac-Leu-Leu-Nle-al	1.6	46.8 ± 1.6
Z-Leu-Leu-Gly-al	40	29.0 ± 3.0
Z-Leu-Leu-Ala-al	3	30.4 ± 0.1
Z-Leu-Leu-Nle-al	0.3	48.4 ± 3.4
Z-Leu-Leu-Ile-al	0.25	50.9 ± 1.4
Z-Leu-Leu-Val-al	0.25	49.5 ± 2.3
Z-Leu-Leu-Nva-al	0.1	43.1 ± 1.6
Z-Leu-Leu-Phe-al	0.05	50.7 ± 2.9
Z-Leu-Leu-Leu-al	0.03	46.2 ± 1.0
Z-Leu-Leu-al	100	18.7 ± 1.2
Z-Leu-Leu-Leu-Leu-al	0.02	34.3 ± 1.5

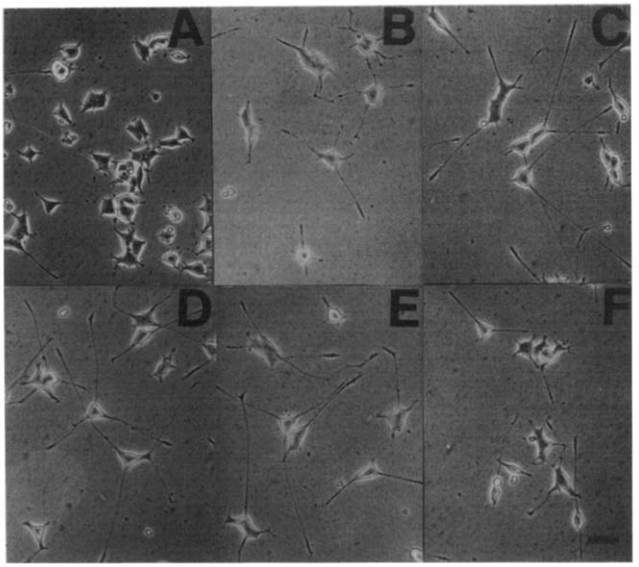

Fig. 1. Phase contrast photomicrographs of PC12h cells grown for 48 h in serum-free N2 medium on collagen-coated dishes. A: no addition. B: 40 ng/ml NGF. C: 1.6 μM Ac-Leu-Leu-Nle-al. D: 0.3 μM Z-Leu-Leu-Nle-al. E: 0.03 μM Z-Leu-Leu-Leu-al. F: 40 μM Z-Leu-Leu-Gly-al. Bar = 35 μm.

We reported that acetyl-Leu-Leu-*Nle*-al, but not acetyl-Leu-Leu-*Met*-al, is an effective neurite initiator [13], so it is possible that the neurite-initiating effect of the tripeptide aldehyde depends on the amino acid residue at the P3 site. We therefore synthesized P3 site-substituted tripeptide aldehydes with the Z group at the N-terminus to investigate the subsite specificity involved in neurite initiation (Table I). Alteration of P3 to a hydrophobic residue (Ile, Val, Nva, Phe, or Leu) produced neurite outgrowth effectively at various concentration (0.03–0.3 μM). The neurites induced by Z-Leu-Leu-X-al (X = Nle, Ile, Val, Nva, Phe, Leu) are similar to those induced by ALLNal in morphological terms, namely one or two long neurites produced from a cell body (Fig. 1C–E). Z-Leu-Leu-Leu-al (ZLLLal) is the most effective for neurite outgrowth, while Z-Leu-Leu-Phe-al (ZLLPal) is the next strongest. ZLLLal induces neurite outgrowth in 50% of cells at a concentration as low as 30 nM, which is 50-fold lower than the concentration of ALLNal required to produce the same effect. We examined the persistance of the neurites induced by ALLNal analogues. In the case of neurites induced by ALLNal, the percentage of cells bearing neurites is maximal after 48 h and then starts to decrease (the percentage of neurite-bearing cells is 46.8%, 39.5%, and 13.0%, respectively, after treatment for 48 h, 72 h and 120 h). When ZLLLal is used, the maximal response is also observed 48 h after treatment, but remains unchanged untill 72 h (the percentage of neurite-bearing cells is 45.0% and 43.5% after

treatment for 48 h and 72 h, respectively) and still 32.5% of the cells has neurites even after 120 h. The same patterns are found for other Z-Leu-Leu-X-al (X = Nle, Ile, Val, Nva, Phe).

Table II summarizes the relationship between the hydrophobicity of the 3rd amino acid residue and that of whole molecule, and the ability to induce neurite outgrowth. The results show that the hydrophobicity of Z-Leu-Leu-X-al is not consistent with the potency for neurite outgrowth. For example, Z-Leu-Leu-Ile-al is the most hydrophobic analogue, but the optimal concentration for neurite outgrowth is 0.25 μM. However, alteration of P3 to a less hydrophobic residue (Gly or Ala) results in much weaker induction, and the neurites produced are not as long as those induced by ALLNal (Fig. 1F). This suggests that a moderate hydrophobicity is important for both neurite initiation and elongation.

To confirm the effect of chain length, deletion or addition of a Leu residue from or to ZLLLal was performed (Table I). Deletion of Leu increased the effective concentration to about 3300-fold, and besides only 20% of cells formed neurites. Addition of Leu had less effect on the effective concentration, but the percentage of neurite-bearing cells was lower than that induced by ZLLLal. These imply that the chain length of three amino acid residues is essential to keep the potency of peptide aldehydes for neurite outgrowth.

Some structure-function relationship has been revealed for ALLNal analogues. A tertiary structure involving both a three peptide chain length and hydrophobicity is necessary for eliciting neurite outgrowth in PC12h cells. Substitution at the P3 site by various amino acid residues causes considerable changes in the optimal concentration for neurite outgrowth. This strongly suggests the existence of a substrate-specific enzyme that participates in neurite outgrowth from PC12h cells.

All of the potent ALLNal analogues for neurite initiation has, more or less, protease inhibitory activity in vitro against, for example, CANP (Tsubuki et al., in preparation). If indeed a target protease is involved in the formation of neurites, what is its localization? Neurite outgrowth induced by tripeptide aldehyde protease inhibitors is not blocked by the concentrated serum-free conditioned medium. Further, Leu-Leu-Leu-al immobilized with Affigel-10, which can not penetrate the cytoplasmic membrane, has no effect on neurite outgrowth in PC12h cells. This suggests that the target protease of ZLLLal is not extracellular. Since the effective tripeptide aldehydes are hydrophobic in nature, they can penetrate the cytoplasmic membrane but may have difficulty diffusing into the cytoplasm. The site of the target protease will therefore be in the peripheral part of the cell, just inside the cell membrane. It has been reported that several nervous systems secrete collagenase-type metalloprotease [11] thrombin-like protease [3] and plasminogen activator [9]; that is, these are all extracellular proteases. These enzymes have been primarily implicated in neurite outgrowth through degradation of extracellular matrix [11]. On the other hand, the target protease of tripeptide aldehyde in PC12h cells may regulate neurite outgrowth from an intracellular position via the proteins lining the membrane or cytoskeleton proteins near the membrane.

In these experiments, we have obtained a powerful tool, ZLLLal, for isolating the target protease. Further characterization of the target protease is essential for elucidating the mechanism underlying the neurite outgrowth of PC12h cells.

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TABLE II

THE RELATIONSHIP AMONG THE 3rd AMINO ACID RESIDUE, HYDROPHOBICITY, AND ABILITY TO INDUCE NEURITE OUTGROWTH IN PC12h CELLS

Optimal concentrations for eliciting the neurite outgrowth are from Table I; hydrophobic parameters for the 3rd amino acid are from Kyte-Doolittle's hydropathy index [7]. Since the C18 column is hydrophobic, the hydrophobicity of Z-Leu-Leu-X-al is directly proportional to peak retention time.

	Gly	Ala	Nle	Ile	Val	Nva	Phe	Leu
Optimal conc. (μM)	40	3	0.3	0.25	0.25	0.1	0.05	0.03
Hydropathy index of the 3rd amino acid (Kyte-Doolittle) [7]	0.4	1.8	–	4.5	4.2	–	2.8	3.8
Retention time (min) of Z-Leu-Leu-X-al from HPLC (C18 column)	3.04	4.17	8.60	9.10	7.41	6.46	7.04	7.62

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