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ISOLATION AND CHARACTERIZATION OF POSSIBLE TARGET PROTEINS RESPONSIBLE FOR NEURITE OUTGROWTH INDUCED BY A TRIPEPTIDE ALDEHYDE IN PC12H CELLS

Yumiko Saito[1]*, Satoshi Tsubuki[2], Hisashi Ito[2], and Seiichi Kawashima[1]

[1] Department of Enzyme Biochemistry, Tokyo Metropolitan Institute of Gerontology, Itabashi-ku, Tokyo 173, Japan

[2] Department of Chemistry, Aoyama-Gakuin University, Setagaya-ku, Tokyo 157, Japan

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SUMMARY: A tripeptide protease inhibitor, benzyloxycarbonyl-Leu-Leu-Leu-aldehyde (ZLLLal), induces the outgrowth of one or two long neurites from PC12 cells. Since this neurite outgrowth is different from that induced by nerve growth factor (NGF) in some aspects, the existence of a molecule that regulates neurite formation in PC12 cells was expected. To identify a target molecule, Leu-Leu-Leu-aldehyde (LLLal) was immobilized as a ligand for affinity chromatography. Proteins of 33-kDa, 35-kDa, and 180-kDa from the membrane and cytoplasmic fractions of PC12 cells bound specifically to the affinity column. ZLLL-COOH has no ability to induce neurite outgrowth, and the 33-kDa, 35-kDa, and 180-kDa proteins do not bind to an LLL-COOH coupled affinity column. By using the LLLal-affinity column, the 33-kDa /35-kDa proteins were found to be converted to 36-kDa/38-kDa proteins during brain development in rats. These results suggest that LLLal-binding proteins are involved in neuronal differentiation.

An important cellular event involved in neuronal development is the extension of neurite. One mechanism that may be responsible for neurite outgrowth is the interaction between extracellular protease and protease inhibitors (1,2). It has been reported that the serine protease inhibitors soybean trypsin inhibitor and leupeptin, as well as glia-derived nexin, enhance neurite outgrowth from neuroblastoma cells and the peripheral nervous system (3,4).

Rat pheochromocytoma cell line, PC12 cells, are induced to differentiate into a phenotype resembling peripheral neurons by neurotrophic factors such as NGF

* To whom correspondence should be addressed.

Abbreviations used: PC12 cells, rat pheochromocytoma cell line; DMEM, Dulbecco's modified Eagle medium; NGF, nerve growth factor; ALLNal, acetyl-leucyl-leucyl-norleucinal; ZLLLal, benzyloxycarbonyl-leucyl-leucyl-leucinal; ZLLL-COOH, benzyloxycarbonyl-leucyl-leucyl-leucine; DMSO, dimethyl sulfoxide; PBS, phosphate-buffed saline.

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(5). This cell line provides an important model for the study of neuronal development and the mechanism of action of certain growth factors (6). Our earlier work has shown that among many protease inhibitors examined, including soybean trypsin inhibitor and leupeptin, only a leupeptin analogue, acetyl-leucyl-leucyl-norleucinal (ALLNal) initiates neurite outgrowth in PC12 cells (7,8). The neurites induced by this inhibitor look different, with a different persistence and a different length, than those that occur when the cells are treated with NGF, dibutyryl cyclic cAMP (dbcAMP), or basic fibroblast growth factor (bFGF) (8). These findings provide evidence that ALLNal and other known neurotrophic factors elicit neurite initiation by different mechanisms and suggest the existence of novel molecules (proteases) that modulate neurite initiation in PC12 cells.

Recently we reported that benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal) is a 50-fold stronger initiator activity of neurite outgrowth than ALLNal (9). To assess the target molecule of ZLLLal, LLLal was immobilized and used as a ligand for affinity chromatography. This procedure resulted in the isolation of 33-kDa, 35-kDa, and 180-kDa proteins which may be responsible for neurite outgrowth in PC12 cells.

MATERIALS AND METHODS

MATERIALS. Wistar rats were from Charles River. Affigel-10 was from Bio-Rad. Nerve growth factor (NGF), 2.5S, was from Takara (Tokyo). S.aureus V8 protease was from Seikagaku-Kogyo (Tokyo). Benzyloxycarbonyl-leucyl-leucyl-leucinal (ZLLLal) and benzyloxycarbonyl-leucyl-leucyl-leucine (ZLLL-COOH) were synthesized according to the method of Ito et al (10).

CELL CULTURE. All procedures were the same as described before (8). In brief, PC12h cells (11) 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 neurite outgrowth, the PC12h cells were plated onto collagen-coated plates with 30nM ZLLLal, 50 uM ZLLL-COOH, or 40ng/ml NGF in serum-free, chemically defined medium for 2 day.

AFFINITY CHROMATOGRAPHY. H-leucyl-leucyl-leucyl-semicarbazone was immobilized to Affigel-10 and the C-terminal group was converted to an aldehyde by acid treatment. PC12 cells were washed twice with PBS. The cells were then ruptured in homogenization buffer (0.1M sodium phosphate/0.2M NaCl/1mM EDTA/1mM EGTA/50 uM leupeptin/100 uM DFP, pH 7.5) and centrifuged at 100,000 x g for 1hr to separate the cytosolic and membrane fractions. The membrane fraction was subsequently resuspended in 1% Triton X-100/homogenization buffer for 1hr at 4°C, and centrifuged at 100,000xg for 1hr. Each fraction was dialyzed against 0.1M sodium phosphate(pH 7.5)/0.2M NaCl for 18hr at 4°C to remove EDTA, EGTA, leupeptin, and DFP, and then applied to the affinity column at 4°C at a rate of 1ml/1min. The column was washed with 0.1M sodium phosphate/0.2M NaCl/1% Triton X-100 (pH 7.5) and 0.1M sodium phosphate/1% Triton X-100 (pH 7.5), and the proteins were eluted first with 0.1M citrate buffer/1% Triton X-100 (pH 4.0) at 4°C, and then with 0.1M sodium phosphate(pH 7.5)/6M urea at room temperature. The eluted proteins were precipitated in 10% trichloroacetic acid and analyzed by SDS-polyacrylamide gel electrophoresis.

The conditioned media from PC12 cell cultures and rat brain homogenates (embryo 16 days, postnatal 1,3,14 days, and 3 months) were processed similarly. Affinity chromatography was done only for the cytoplasmic fractions of liver, kidney, spleen, heart, and muscle of 3 month old rats.
Other procedure - Peptide mapping of the isolated proteins was performed in gels containing SDS using S.aureus V8 protease as described by Cleveland et al (12). Protein determination was by the method of Lowry et al (13).

RESULTS

Since ZLLLal is a very potent inducer of neurite outgrowth in PC12 cells , an Affigel-10 affinity resin with Leu-Leu-Leu-al (LLLal) immobilized through the amino terminus was prepared. ZLLL-COOH, in which the C-terminal group is not converted to a reactive aldehyde group, does not cause neurite outgrowth. That is, after 48hr in cultures fed with 50 uM ZLLL-COOH, 1.8 ± 0.2 % (mean ± SEM) of the total cells had neurites longer than 35 uM compared with 46.2 ± 2.4 % for 30nM ZLLLal-treated cells . Therefore, immobilized Leu-Leu-Leu-COOH was used to examine non-specific binding.

When the cytoplasmic fraction of PC12 cells was applied to the LLLal affinity column and the bound proteins were eluted with citrate buffer (pH 4.0) followed by 6M urea/sodium phosphate buffer (pH 7.5), 33-kDa, 35-kDa, and 180-kDa proteins were observed on SDS-polyacrylamide gel electrophoresis. Other bound proteins also bound to immobilized Leu-Leu-Leu-COOH and thus might bind only by hydrophobic interaction. The proteins eluted with citrate buffer (pH 4.0) were found to be richer in the 33-kDa than the 35-kDa species, while the 180-kDa protein was effectively eluted with 6M urea (pH 7.5) (Fig.1). Elution with 0.1% SDS after 6M urea (pH 7.5) showed that no proteins remained bound to the affinity column. The 33-kDa, 35-kDa, and 180-kDa proteins did not elute when the ionic strength (0-1.5M NaCl) or the pH (pH 9.0) was changed. The 33-kDa, 35-kDa, and 180-kDa proteins were also found in the membrane fraction, but the amounts per cell were smaller than in the cytoplasmic fraction (Fig.1). When conditioned media from the PC12 cell culture were applied to the LLL-COOH and the LLLal affinity columns, no specific binding of any proteins, including the 33-kDa, 35-kDa, and 180-kDa proteins, was detected (data not shown).

The 33-kDa and 35-kDa proteins that elute from the affinity column at low pH were the focus of an attempt to purify and identify the target molecules of ZLLLal. Because PC12 cells are derived from rat, the cytosol fractions from the liver, heart, muscle, spleen, kidney, and brain of rats (3 months) were applied to the LLLal affinity column, and the column was eluted with citrate buffer (pH 4.0). Fig.2 shows the eluted proteins detected by SDS-polyacrylamide gel

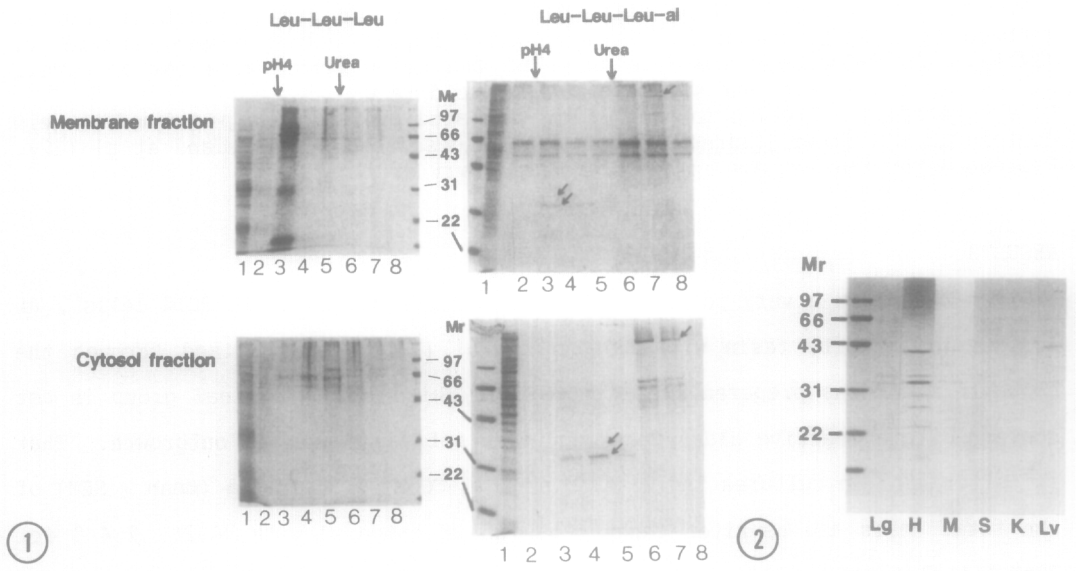

FIG.1.
LLL-COOH and LLLal affinity chromatographies of PC12 cell homogenates.
The cytosol fraction (2mg protein) or membrane fraction (3 mg protein)of PC12 cells was charged to an LLL-COOH or LLLal Affigel-10 affinity column (4.5 µmol of bound LLL-COOH or LLLal/column). The columns were washed with 0.1M sodium phosphate (pH 7.5)/0.2M NaCl/1% Triton X-100 and 0.1M sodium phosphate (pH 7.5)/1% Triton X-100, and the proteins were eluted with 0.1M sodium citrate (pH 4.0)/1% Triton X-100 (5 fractions of 1ml each). These operations were performed at 4°C. Subsequently, the affinity columns were washed with 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 and the pH was returned to neutral. The column was then treated with 6M urea/0.1M sodium phosphate (pH 7.5) and 6 fractions of 1ml each were collected. The eluted proteins were precipitated in 10% trichloroacetic acid and analyzed by SDS polyacrylamide gel electrophoresis.

Lane 1: charged proteins
2: 0.1M sodium phosphate (pH 7.5)/1% Triton X-100 eluates
3-5: 0.1M sodium citrate (pH 4.0)/1% Triton X-100 eluates (fractions 1-3, respectively)
6-8: 6M urea/0.1M sodium phosphate (pH 7.5) eluates (fractions 1-3, respectively)

Arrows indicate the 33-kDa, 35-kDa, and 180-kDa proteins that bound specifically to the LLLal affinity column.

FIG.2.
LLLal affinity chromatography of cytosol fractions from adult (3 months) rat organs.
Cytosol fractions (80mg protein) from adult rat lung (Lg), heart (H), muscle (M), spleen (S), kidney (K), and liver (L) were charged to the LLLal Affigel-10 column (9 µmol of bound LLLal/column). Washing, elution at low pH, and electrophoresis of combined fractions 1 and 2 of the low pH eluate were performed as described for FIG.1.

electrophoresis. Appreciable amounts of the 33-kDa/35-kDa proteins were identified in heart and muscle. When the cytosolic fraction from postnatal 1 day rat heart was applied and eluted with low pH, the 33-kDa and 35-kDa proteins were identified at levels comparable to those in adult rat heart (data not shown). These proteins did not bind to the LLL-COOH affinity column.

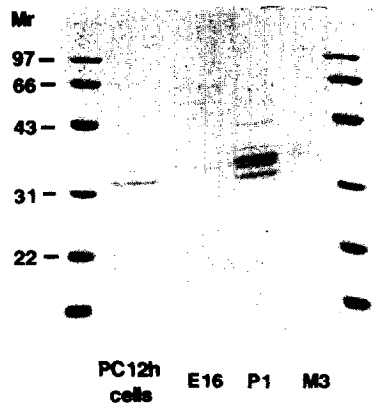

FIG.3.
LLLal affinity chromatography of the cytosol fractions from PC12 cells and rat brain.
Cytosol fractions from PC12 cells (10mg protein), embryonic 16 day brain (E16, 10mg protein), postnatal 1 day brain (P1, 10mg protein), and 3 month brain (M3, 30mg protein) were charged to the LLLal Affigel-10 affinity column (4.5 μmol of bound LLLal/column). Washing, elution with 0.1M sodium citrate (pH 4.0)/1% Triton X-100, and electrophoresis of the combined fractions 1 and 2 of the low pH eluate were performed as described for FIG.1.

Proteins eluted from the LLLal affinity column at low pH were investigated for their appearance during brain development in the rat (embryonic 16 days, postnatal 1, 3, 14 days, and 3 months). Fig.3 and Table 1 show the pattern of the eluted proteins detected by SDS-polyacrylamide gel electrophoresis. As a proportion of the total protein applied to the column, the amount of the 33-kDa proteins was very low in 16 day embryos, but rapidly increased to maximal levels 1-3 days postnatally, and then decreased from 14 days postnatally. The amount of the 35-kDa protein was greater than that of the 33-kDa protein in 16 day embryos, in contrast to the situation in PC12 cells, but the level decreased from 14 days postnatally. From 1 day postnatally, the 36-kDa/38-kDa proteins began to appear, at which time the proteins were present in the order: 36-kDa > 33-kDa, 35-kDa > 38-kDa. After about 14 days, the amount of the 33-kDa/35-kDa proteins decreased as mentioned above, and the amount of 36-kDa/38-kDa proteins gradually decreased until, finally, only small amounts could be detected in adult brain. A similar transition of the 33-kDa, 35-kDa, 36-kDa, 38-kDa proteins was observed in the membrane fraction during brain development.

Fig.4 shows the peptide maps of the 33-kDa proteins derived from PC12 cells, 1 day postnatal rat brain, and 3 month rat muscle after limited proteolysis by S.aureus V8 protease. Since the same S.aureus V8 protease digestion fragments can be seen despite the tissue source, the 33-kDa proteins isolated from 1 day

TABLE 1
Proteins eluted from the LLLal affinity column at low pH (pH 4.0)

		Rat brain			
	PC12 cell	Embryonic 16days	Postnatal 1day	Postnatal 14days	Postnatal 3months
33K	◎	△	◎	△	△
35K	○	○	◎	△	△
36K	×	×	◎	◎	○
38K	×	×	○	○	○

×: undetected △: faint ○: medium ◎: strong

Cytosol fractions were charged to the LLLal affinity column. The column was washed successively with 0.1M sodium phosphate (pH 7.5)/0.2M NaCl/1% Triton X-100 and 0.1M sodum phosphate (pH 7.5)/1% Triton X-100, and the proteins were eluted with 0.1M sodium citrate (pH 4.0)/1% Triton X-100. Eluted proteins were analyzed by SDS-polyacrylamide gel electrophoresis. Symbols indicate qualitatively the degree of protein staining by Coomassie Brilliant Blue.

postnatal rat brain and 3 month rat muscle are thought to be the same as the 33-kDa protein isolated from PC12 cells.

DISCUSSION

Our results show the isolation and characterization of target proteins possibly involved in neurite outgrowth initiated by Z-Leu-Leu-Leu-aldehyde

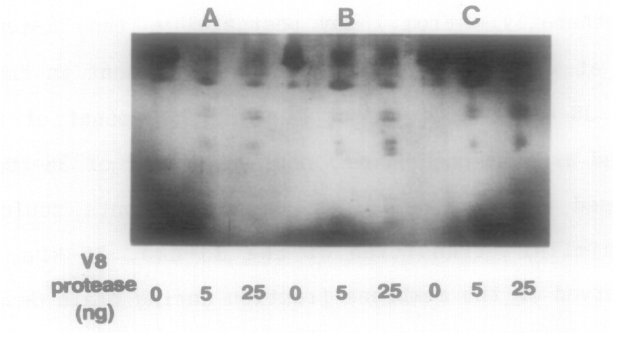

FIG.4.
Peptide maps of the 33K protein derived from PC12 cells, postnatal 1 day rat brain, and adult (3 months) rat brain.
Peptide mapping was performed as described by Cleveland et al. (12) with the indicated concentrations of S.aureus V8 protease . A: the 33K protein derived from PC12 cells; B: the 33K protein derived from postnatal 1 day rat brain, C: the 33K protein from 3 month rat brain.

(ZLLLal) in PC12 cells. The molecular weights of the proteins isolated from the cytoplasmic and membrane fractions of PC12 cells are 33-kDa, 35-kDa, and 180-kDa. Z-Leu-Leu-Leu-COOH does not cause neurite outgrowth, even at 50 uM, and the 33-kDa, 35-kDa, and 180-kDa proteins do not bind to a Leu-Leu-Leu-COOH affinity column. Thus, all three proteins are likely to be involved in neurite outgrowth.

The 33-kDa, 35-kDa, and 180-kDa proteins are not isolated from conditioned medium of PC12 cells. We previously reported that concentrated conditioned medium does not block neurite outgrowth induced by the protease inhibitor ALLNal (8), and that LLLal immobilized to Affigel-10, so it can not penetrate the cytoplasmic membrane, has no effect on neurite outgrowth in PC12 cells (9). These results also suggest that the target molecules of ZLLLal are intracellular rather than extracellular. This is quite different from the situation with other proteases involved in neurite outgrowth such as plasminogen-activator (14), thrombin-like protease (15), or metalloprotease (2), which are located extracellularly. ZLLLal is very hydrophobic in nature, so it can penetrate the cytoplasmic membrane, interact with target proteins, and finally induce a link for neurite initiation.

In rat brain, the 33-kDa and 35-kDa proteins were found to switch to 36-kDa and 38-kDa proteins during development (Fig.3, Table 1). This developmental transition is not seen in non-brain tissue, i.e. heart. A larger amount of the 33-kDa, 35-kDa, 36-kDa, and 38-kDa proteins as a proportion of the applied protein was observed from 1 to 14 days postnatally in brain. The time from 1 to 14 days postnatally is coincident with the period of most active neurite outgrowth in rat cerebrum. This result suggests that the 33-kDa/35-kDa proteins are involved in neuritogenesis of PC12 cells, as well as in <u>in vivo</u> brain neuritogenesis. The same 33-kDa protein was identified in heart and skeletal muscle. In these tissues, the nervous system (synapse) is well-developed and the involvement of the protein in neuritogenesis is conceivable.

We tried to identify protease activity in the 33-kDa, 35-kDa, and 180-kDa proteins using alkalin-denatured casein as a substrate and by zymography in gelatin, but none could be detected. This may be due to irreversible denaturation of any activity. This possibility is supported by the fact that on reapplication to a new LLLal affinity column after neutralization of the 33-kDa/35-kDa protein fraction or removal of urea from the 180-kDa protein fraction, the proteins no longer bind. ALLNal strongly inhibits the activity

of the cysteine protease cathepsin L (16). Therefore, cathepsin L is regaeded as a possinble target molecule of the protease inhibitor that induces neurite outgrowth in PC12 cells. However, the 33-kDa, 35-kDa, and 180-kDa proteins do not react with antiserum specific for cathepsin L antiserum (17) on immunoblotting.

Since the 33-kDa protein derived from PC12 cells and rat muscle are the same , the 33-kDa protein can be isolated in large quantities from rat muscle by LLLal affinity chromatography. Further studies are in progress to identify the 33-kDa protein by determination of its internal amino acid sequence, and to elucidate the molecular mechanism underlying neurite outgrowth induced by ZLLLal.

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