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Purification and Characterization of a 15 kDa Protein (p15) Produced by *Helicosporium* That Exhibits Distinct Effects on Neurite Outgrowth from Cortical Neurons and PC12 Cells

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We have found that a 15 kDa protein (p15) produced by a fungus of *Helicosporium* genus induced characteristic morphological changes on neurites extending from rat cerebral cortex neurons in culture. In the presence of p15, neurite elongation from cortical neurons was markedly inhibited, and they extended short, slender, and less branched neurites. Laminin-induced promotion of neurite outgrowth was also dramatically suppressed. In contrast, p15 promoted nerve growth factor (NGF)-induced neurite outgrowth from PC12 cells, although treatment of cells with p15 alone had little effect. Partial amino acid sequence analysis of p15 revealed that it had limited homology to plant lectins. These results suggest that the mechanism of neurite outgrowth was considerably different between cortical neurons and PC12 cells and that p15 modulated neurite outgrowth in cell type-specific manners.

During the development of vertebrate nervous system, neurons receive a variety of chemosensory signals from the environment, which are secreted in the extracellular milieu or expressed on the surface of other neurons and glial cells as membrane-bound molecules. Using these molecules as cues, neurons extend neurites over long distances toward correct targets to eventually form synaptic connections. These chemosensory signals are mainly classified into two categories, one of which consists of proteins with neurite-growth promoting activity (1-4), and the other consists of inhibitors of neurite extension (5-10). In some cases, however, a single protein exhibits both of these activities depending on the cell types examined (7, 11).

In an attempt to understand the detailed mechanisms of neurite outgrowth, we have been employing an approach in which we searched from microbial secondary products for novel inhibitors or activators of neurite outgrowth and then investigated the molecular mechanism of actions of those compounds. In this paper, we report purification and characterization of p15, a novel protein produced by a fungus of *Helicosporium* genus, which has distinct effects on neurite outgrowth from cultured cortical neurons and from NGF-treated PC12 cells.

MATERIALS AND METHODS

Cell culture. Primary culture of rat cerebral cortex neurons was obtained from embryonic day 18 fetuses as described (12). Briefly, cerebral cortex was dissected under microscope, then cells were suspended by papain treatment and cultured in Dulbecco's modified Eagle's medium (DMEM; high glucose type) supplemented with 5% fetal calf serum (FCS), 5% horse serum, 5 μg/ml insulin, 5 μg/ml transferrin, 1mM sodium pyruvate in a humidified 10% CO_2 atmosphere. When the effects of various samples were examined, cells were plated to the 96-well plate at the cell density of 1.24×10^5 cells/cm^2, and the samples were added simultaneously. The morphology of cells was observed

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Abbreviations: bFGF, basic fibroblast growth factor; FCS, fetal calf serum; NGF, nerve growth factor; PAGE, polyacrylamide gel electrophoresis; PEI, polyethylenimine; SDS, sodium dodecyl sulfate.

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after 24 hr. The culture plates were coated with polyethylenimine (PEI; 0.2% in sterile water) for overnight and were washed three to four times with phosphate-buffered saline. When the culture plates were coated with laminin, PEI-coated plates were treated with laminin (100 μg/ml) for 2 hr at 37 ℃ and were similarly washed.

Subclone PC12-22a, isolated from parental rat pheochromocytoma PC12, was kindly provided from Dr. Takashima of Mitsubishi-kagaku Institute of Life Sciences, Tokyo, Japan. They were cultured in DMEM supplemented with 5% FCS and 5% horse serum as described (13). When PC12-22a cells were treated with NGF or bFGF, cells were cultured in the PEI-coated 96-well plates at the density of 3×10^3 cells/cm^2 for 24 hr to allow cells to adhere to the culture plates. Then 50 ng/ml NGF or 5 ng/ml bFGF and samples were added simultaneously. The morphology of the cells was observed 24 hr after addition of NGF and samples.

Fermentation of fungus H16636 and purification of p15. The strain H16636 that produced active substances was precultured in flask containing seed media (4% saccharose, 2% pharmamedia, 2% dry yeast, 1% polypeptone, 0.2% K_2HPO_4, 0.2% $CaCO_3$, 0.1% Tween 80) on rotary shaker at 25 ℃, 120 r.p.m. for 8 days. Then the cells were reseeded in 5-liter flasks containing 1.5 liter of production media (3% soluble starch, 1% peanuts powder, 1% soybean meal, 1% KH_2PO_4, 1% NaH_2PO_4) and incubated on rotary shaker at 25 ℃, 120 r.p.m. for further 8 days. From 12 liters of culture broth, mycelium was collected by centrifugation and extracted with 70% acetone. Acetone was removed *in vacuo*, and the extract was applied to a Dowex 50W-X4 column (H^+-type). After washing with water, the active material was eluted with 0.5N ammonia water. The eluate was concentrated *in vacuo*, and was lyophilized. The dried powder was dissolved in 20% methanol-water, applied to a Toyopearl HW40 gel filtration column, and was eluted with 20% methanol-water. The active fraction was lyophilized and was obtained as 100 mg of crude brown powder. The crude powder was dissolved in water, applied to two sequential steps of gel filtration column, Sephadex G-100 and Toyopearl HW55, and the purified active fraction was obtained as 10 mg of brown powder.

SDS-PAGE and other analytical procedures. Samples were dissolved in SDS sample containing 50 mM Tris-HCl (pH 6.9), 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and boiled for 2 min. and then subjected to SDS-polyacrylamide gel electrophoresis (PAGE) containing 15% acrylamide according to Laemmli (14) with slight modifications. Partial N-terminal amino acid sequences of p15 blotted onto the PVDF membrane were determined as described (15) with gas-phase amino acid sequencer ABI model 473A (Applied Biosystems Inc., USA).

RESULTS

To search for novel active compounds that have modulatory effects on neurite outgrowth, neural network formation and neuronal survival, we screened microbial culture broth by examining microscopically their effects on the morphology of neurites from rat cortical neurons in culture. Among 4000 samples of fungi and 2000 samples of *Actinomycetes* tested, we found a fungus, named H16636 which was identified as a genus of *Helicosporium*, produced a substance that inhibited neurite outgrowth and induced characteristic morphological changes of neurites without influencing the viability of neurons.

Purification of the active compound was performed as described in Materials and Methods. As this active fraction lost its activity with 12 hr of proteinase K treatment (data not shown), we supposed that the active compound might be a protein and therefore analyzed it by SDS-PAGE. As shown in Fig. 1, a protein with its apparent molecular weight of 15 kDa which was barely detectable in the partially purified fraction (lane 1) was concentrated in the final sample (lane 2). When this final sample was run on the native polyacrylamide gel electrophoresis, the extract from the gel position corresponding to the 15 kDa protein had activity toward cortical neurons. Therefore we concluded that this 15 kDa protein was the active compound and thus termed it as p15.

The effect of p15 on cultured rat cerebral cortical neurons isolated at embryonic day 18 was examined (Fig. 2). In the control, nearly 60%Å of cells extended neurites with longer than twice the length of the cell diameter during the first 24 hr in the culture. In contrast, cells treated with p15 at 5 μg/ml from the onset of culture were markedly inhibited to grow neurites; the proportion of cells with neurites longer than twice the cell diameter decreased to less than 20% of total cells, and more than half of the cells extended neurites no longer than one cell diameter. The morphology of neurites extending from these cells was apparently different from the control cells. They were slender and branch formation was suppressed. The inhibitory effect of p15 on neurite outgrowth was much more evident when cells were cultured on laminin-coated culture plates. When the cortical neurons were grown on laminin-coated culture

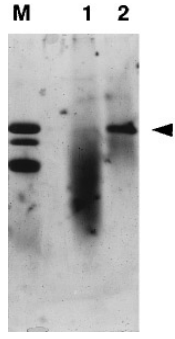

FIG. 1. SDS-PAGE analysis of purified p15. Aliquots of samples from Toyopearl HW40 column (lane 1) and the final preparation from Toyopearl HW55 column (lane 2) were analyzed by SDS-polyacrylamide gel electrophoresis and were stained by coomassie brilliant blue. The positions of the low molecular weight marker proteins are indicated in the left (lane M; from the top, 16.9, 14.4, and 10.7 kDa, respectively), and the position of p15 is indicated by arrowhead in the right.

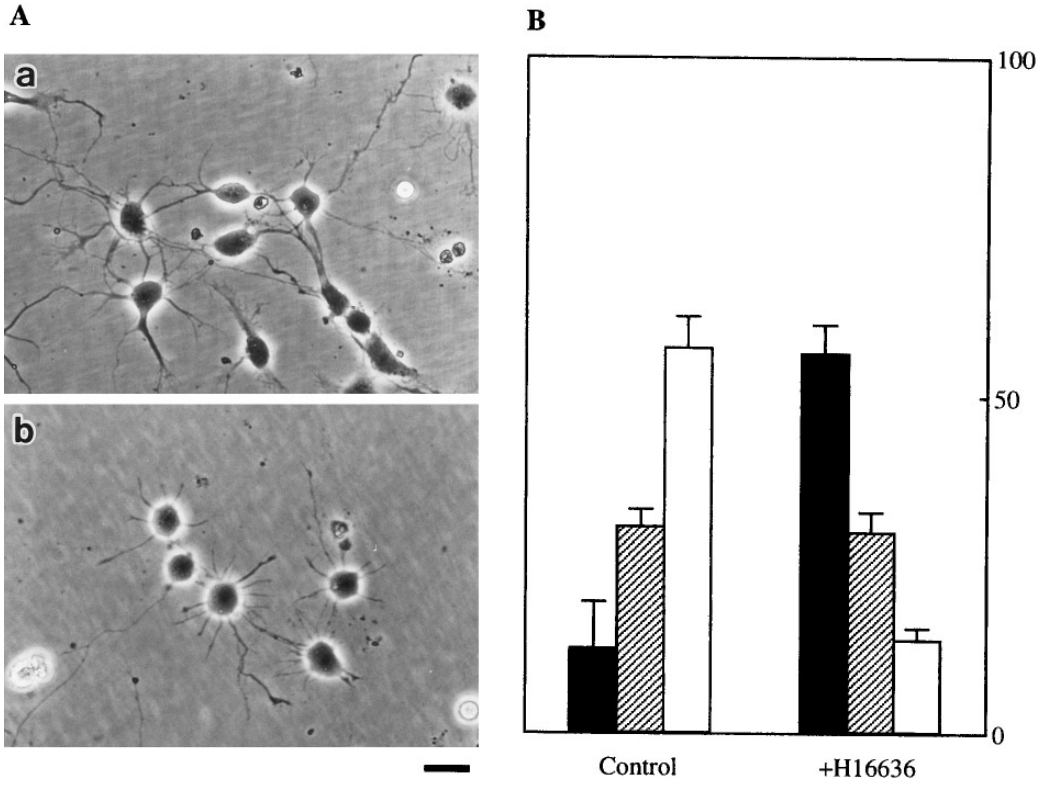

FIG. 2. The effect of p15 on cerebral cortex neurons. (A) Cerebral cortex neurons were plated at 1.24×10^5 cells/cm^2 onto PEI-coated 96-well plates and cultured for 24 hr. Purified p15 was added to the cells at the beginning of the culture. In the control (a), cells extend long, branched neurites, while cells treated with p15 extend shorter, less branched neurites (b). (B) Quantitation of neurite outgrowth. The percentage of cells on which all neurites were shorter than the cell diameter (black), the longest neurites were longer than the cell diameter but shorter than twice the cell diameter (hatched), and the longest neurites were longer than twice the cell diameter (white) are shown. Data are mean values +/−SE for three independent experiments, in each of which more than 100 cells in random fields were counted. Significance was attributed to $P<0.01$. Bar, 20 μm.

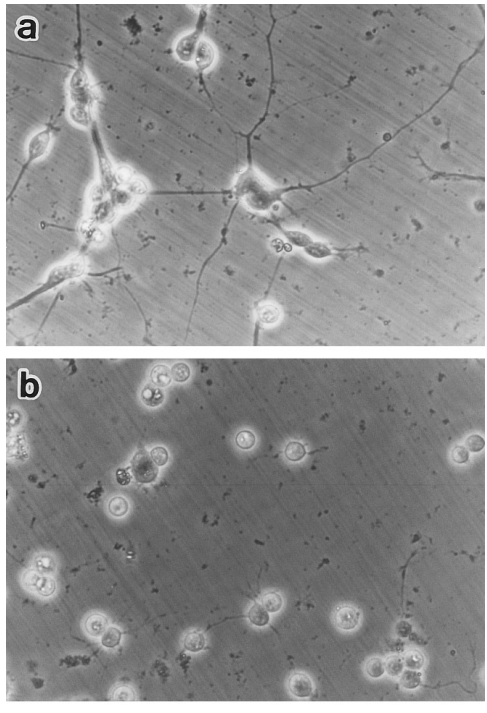

FIG. 3. The effect of p15 on cerebral cortex neurons cultured on laminin-coated substratum. Cortical neurons grown on culture plates coated with laminin (100 μg/ml) tended to aggregate, and from these aggregates they extended neurites significantly longer than those from cells on PEI-coated plates (a). In contrast, neurite outgrowth was dramatically suppressed in the presence of p15 (b). Bar, 20 μm.

plates, the neurite outgrowth was greatly enhanced compared with that on PEI-coated plates (Fig. 3 a, compare with Fig. 2A). In the presence of p15, neurite outgrowth was inhibited and promotive activity of laminin on neurite outgrowth was abolished (Fig. 3 b).

We then tested the effects of p15 on NGF-stimulated neurite formation from rat pheochromocytoma cell line PC12-22a. In contrast to the inhibitory effects on the cortical neurons, p15 promoted neurite outgrowth from PC12-22a cells when added with optimum concentration (50 ng/ml) of NGF for 24 hr (Fig. 4 and Table 1). When p15 was added alone or with lower concentration of NGF (5 ng/ml) at which NGF could not induce neurites, the promotive effects of p15 was not observed. Similar stimulatory effect of p15 on neurite outgrowth from PC12-22a cells was observed by co-treatment with basic fibroblast growth factor (bFGF; data not shown).

Purified p15 was blotted onto PVDF membranes and its partial N-terminal 22 amino acid sequences were determined (Fig. 5). Since the first amino acid determined was alanine, it is likely that p15 was processed from a putative larger precursor. Homology search of amino acid databases revealed that p15 was a novel protein, but limited homology with concanavalin A from *Canavalia gradiata* and lectin a chain from *Dioclea grandiflora* was detected (Fig. 5; 50% identity in 12 amino acids overlap) (16, 17).

DISCUSSION

In this report, we have examined the effects of p15, a protein produced by a fungus of *Helicosporium* genus, on neurite outgrowth from from cortical neurons and from PC12-22a

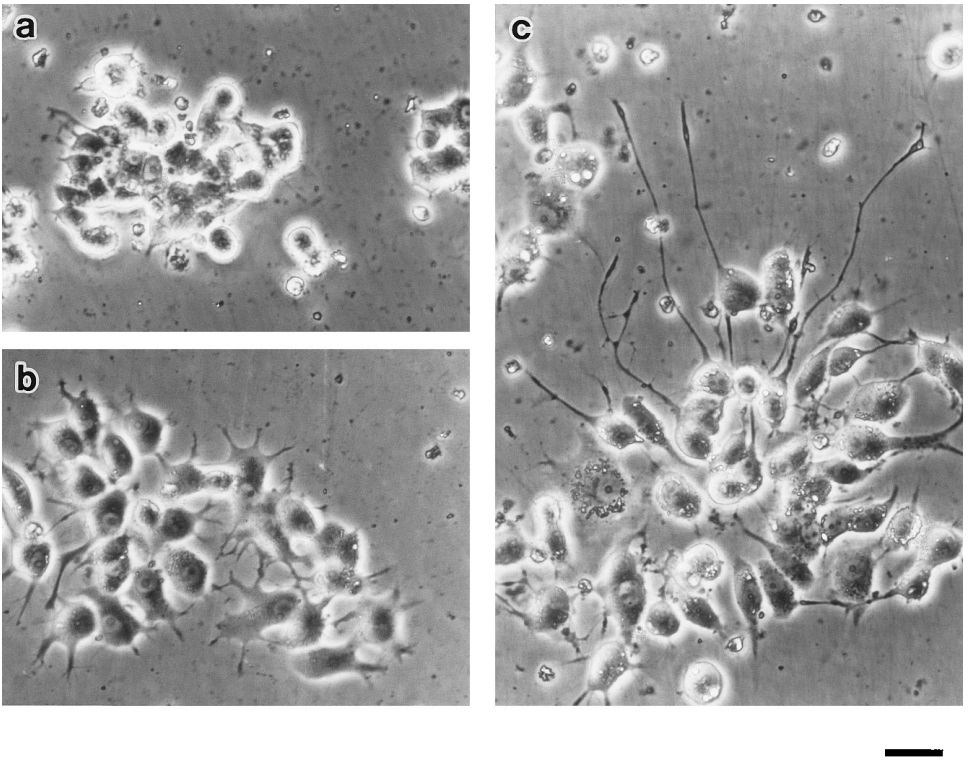

FIG. 4. p15 stimulates NGF-induced neurite outgrowth from PC12-22a cells. PC12-22a cells were cultured in the absence (a) or presence (b) of NGF (50 ng/ml) or NGF plus 5 μg/ml of p15 (c) for 24 hr. In the culture treated with NGF alone, cells extended only minor neurites, which were slightly longer than the neurites of untreated cells. In contrast, cells treated with NGF plus p15 extended markedly longer neurites, the length of which was sometimes several times as long as the diameter of the cell bodies.

cells. In the presence of p15 neurite outgrowth from cortical neurons were markedly inhibited, and the characteristic morphological changes of neurites were induced. In addition, cell bodies of p15-treated cells were somewhat round-shaped and were more refractive under the phase contrast microscopy than those of control neurons. This might indicate that p15-treated cells became less-adhesive to the substratum, but this possibility was not further examined.

Although we tested a variety of drugs including inhibitors of kinases and phosphatases, cytoskeletal components, macromolecular synthesis, protein transport and so on, the effect of

TABLE 1
The Stimulatory Effect of H16636 on Neurite Outgrowth from NGF-Treated PC12-22a Cells

	Experiment 1	Experiment 2	Experiment 3
NGF alone	25.0 (33/132)	22.9 (33/144)	22.2 (35/158)
NGF + H16636	46.6 (69/148)	43.1 (65/151)	40.6 (71/175)

PC12-22a cells were treated with 50 ng/ml NGF in the presence or absence of H16636 at 5 μg/ml. After 24 hr, the cells were fixed and the numbers of cells with neurites longer than 20 μm were counted. The results from three independent experiments were expressed as the percentages of such cells in the total numbers of cells. In parentheses, the actual numbers of cells with 20 μm $<$ long neurites/total cells were shown. Significance was attributed to $P < 0.01$.

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Concanavalin A: 75 SPQGSSVGRALFYAPVHIWESS
                                *** *   **
          p15:  1 ATIEQTTDTLLFYTPIAQWEAA
                                *** *   **
Lectin α chain:164 DPQGNSVGRALFYAPVHIWESS
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FIG. 5. Partial N-terminal amino acid sequences of p15. Partial N-terminal amino acid sequence of p15 was determined and was shown in the top. Amino acid sequences of concanavalin A from *Canavalia gradiata* (middle) and lectin α chain from *Dioclea grandiflora* (bottom) were derived from database search (accession numbers A34139 and JU0176, respectively) and were compared with those of p15, with identical residues indicated by asterisks. Amino acids are shown by single letters, and the numbers from the N-termini are indicated at the left.

p15 was distinct from any of these known drugs. The effect of p15 was cytostatic, since cortical neurons were viable even after 48 hr treatment by p15, as tested by tripan blue staining (data not shown).

In contrast to inhibiting neurite outgrowth from brain neurons, p15 promoted neurite outgrowth from NGF-treated PC12-22a cells (Fig. 4). Similar results have been reported in which a single molecule exhibits opposite activities depending on the cell types examined. For example, myelin-associated glycoprotein (MAG), a member of the immunoglobulin superfamily, had been reported to be inhibitory to neurite outgrowth from rat cerebellar neurons, but promotive to that from postnatal rat dorsal root ganglion (DRG) neurons (11). Collapsin, which was found to cause growth cone collapse and paralysis of chicken DRG neurons, was shown to be inactive to the neurons of retinal ganglion cells (7). Such specificity might be derived from the cell-type specific recognition mechanisms of axonal guidance pathway.

Since p15 *per se* was unable to induce neurites without or in the presence of low concentration of NGF (5 ng/ml), it seemed unlikely that p15 was involved in direct activation of NGF signaling pathway, but rather was involved in augmentation and stabilization of signals generated by NGF. Length of neurites after treatment for 48 hr with p15 plus 50 ng/ml NGF did not significantly differ from that with NGF alone, suggesting that p15 might work in the early step of differentiation of PC12-22a cells and accelerated the rate of neurite formation and outgrowth. Consistent with this was that p15 had no activity on PC12-22a cells when added 24 hr after the culture had started.

Partial N-terminal amino acid sequence analysis suggested that p15 was a novel protein (Fig. 5). Limited homology was found with plant lectin concanavalin A (Con A) and lectin a chain from *Dioclea grandiflora* (16, 17). It has been reported that Con A has stimulatory effects on neurite outgrowth from various neuronal cell types, such as chick dorsal root ganglion neurons and neurons from leech and *Aplysia* (18). This result suggests that clustering of specific cell surface molecules by carbohydrate-binding proteins activates second messenger pathways which eventually leads to neurite outgrowth. Con A itself, however, had little effect on neurite outgrowth from rat cortical neurons, and the region of Con A with homology to p15 was different from its estimated carbohydrate-binding region (19). Further analysis is required to elucidate the mechanism in which p15 promoted neurite outgrowth from NGF-or bFGF-treated PC12-22a cells.

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