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Characterization of a Novel Fungal Protein, p15, Which Induces Neuronal Differentiation of PC12 Cells[1]

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In our previous paper, we reported that a 15 kDa protein (p15) produced by a fungus, genus *Helicosporium*, enhanced NGF-induced neurite outgrowth from PC12 cells. Here we further characterized the actions of p15. The complete amino acid sequence of p15 was determined and it was shown to be a hydrophilic protein composed of 118 amino acid residues with two intramolecular disulfide bridges. p15-induced neurite outgrowth was blocked by the depletion of extracellular Ca^{2+} in the culture medium and was significantly inhibited by L-type Ca^{2+} channel inhibitor nicardipine. p15 stimulated Src kinase and MAPK activities, and neurite outgrowth was not observed in srcDN2, a dominant negative c-*src*^{K295R}-expressing cell line, and was significantly reduced in RasN17-expressing cells. These results suggest that p15 stimulates neurite outgrowth through the potentiation of L-type Ca^{2+} channels, thereby activating the Src-Ras-MAPK cascade.

Key words: depolarization, L-type Ca^{2+} channels, neurite outgrowth, PC12, Src kinase.

Rat pheochromocytoma cell line PC12 cells exhibit the phenotype of sympathetic neurons in response to nerve growth factor (NGF) including extension of neurites and expression of various neuronal proteins (*1, 2*). NGF binds to its receptor, Trk, and triggers its tyrosine kinase activity (*3, 4*). Autophosphorylation of Trk on tyrosine residues provides docking sites for the Src homology-2 (SH2) domains of adapter protein Shc (*5*), which binds and activates Grb2/Sos, thereby activating Ras (*6-8*). Ras activation results in activation of mitogen-activated protein kinase (MAPK) (*9-11*), which is thought to be necessary and sufficient for PC12 differentiation (*12, 13*).

Ca^{2+} is a critical mediator of signaling in neurons, and affects a variety of neuronal responses such as gene expression, differentiation, survival and neuronal plasticity (*14, 15*). Cytosolic Ca^{2+} in resting cells is low (~100 nM), but extracellular Ca^{2+} at millimolar concentrations can rapidly enter neurons in response to ligand stimulation or membrane depolarization through ligand-gated or voltage-sensitive Ca^{2+} channels (VSCCs). L-type Ca^{2+} channels, one type of VSCCs which is localized to cell bodies and the bases of proximal dendrites in neuronal cells, have been shown to act in regulating Ca^{2+}-dependent signaling events in postsynaptic neurons. For example, Ca^{2+} influx through L-type Ca^{2+} channels plays a major role in regulating gene expression in primary cortical neurons (*16, 17*). L-type Ca^{2+} channels are also expressed in PC12 cells and have been suggested to mediate the induction of gene expression in response to membrane depolarization (*18*). Recently, it was shown that voltage-gated Ca^{2+} influx can activate the Ras/MAPK cascade, suggesting that the Ca^{2+} and growth factor signaling pathways overlap (*19*). Furthermore, Ca^{2+} influx through L-type Ca^{2+} channels increases Src kinase activity, and induces the formation of the Shc/Grb2 complex, thereby activating the Ras/MAPK signaling pathway (*20, 21*). Several other mechanisms have been proposed to account for the activation of Ras by Ca^{2+}, including the involvement of tyrosine kinase PYK2, phosphorylation of epidermal growth factor receptor, and RasGRF, a calmodulin-stimulatable guanine-nucleotide exchange factor for Ras (*20-22*).

Previously, we isolated a 15 kDa protein, p15, from the culture broth of a fungus, genus *Helicosporium*, by screening for regulatory molecules for neurite outgrowth (*23*). p15 markedly promoted nerve growth factor (NGF)-induced neurite outgrowth from PC12 cells, although p15 itself failed to induce neurites in the absence of NGF. In contrast, neurite elongation from rat cortical neurons in culture was remarkably affected by p15; they had short,

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Abbreviations: BAPTA/AM, 1,2-bis(2-aminophenoxy)-ethane-*N,N,N,N*-tetraacetic acid acetoxymethyl ester; CNBr, cyanogen bromide; CPT-cAMP, 8-(4-chlorophenylthio)adenosine 3′:5′-cyclic monophosphate; DMEM, Dulbecco's modified Eagle's medium; DRG, dorsal root ganglion; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethansulfonic acid; HRP, horseradish peroxidase; MBP, myelin basic protein; MAPK, mitogen-activated protein kinase; NF, neurofilament; NGF, nerve growth factor; PEI, polyethyleneimine; PMSF, phenylmethylsulfonyl fluoride; RP, reverse-phase; TFA, trifluoroacetic acid.

slender and less branched neurites compared with those of normal cortical neurons. Thus, p15 modulates neurite outgrowth in a cell type-specific manner, suggesting that the mechanism underlying neurite outgrowth is different between PC12 cells and cortical neurons.

In this study, we have further studied the action of p15. We first determined the complete amino acid sequence of p15 by sequencing the overlapping peptide fragments obtained on chemical and enzymatic degradation of purified p15. We found that the Ca^{2+} signaling pathway is involved in p15-stimulated neurite outgrowth; neurite-inducing activity of p15 was blocked by the depletion of extracellular Ca^{2+} in the culture medium and was significantly inhibited by L-type Ca^{2+} channel inhibitor nicardipine. p15 induced the Src kinase activity to a similar extent with depolarization stimuli, while NGF did not induce the increase in Src kinase activity. A PC12 subline that stably expressed the dominant-interfering form of Src (srcDN2) was defective in p15- or depolarization-induced neurite outgrowth, although treatment with NGF caused neurite outgrowth. In addition, p15 was unable to induce neurites in a dominant negative Ras-expressing mutant, and p15 induced a transient increase in MAPK activity. These results suggest the involvement of Src and MAPK in p15-induced neurite outgrowth from PC12 cells. We suggest that p15 exerts its effect by activating L-type Ca^{2+} channels, thereby activating a downstream signaling cascade.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials and chemicals used in this work were as follows: endoproteinase Asp-N from Boehringer Mannheim (Mannheim, Germany); cyanogen bromide from Wako Pure Chemical (Osaka); and BNPS-skatole from Pierce Chemical (Rockford, IL). Other chemicals used were of analytical grade.

Cell Culture and Assessment of Neurite Outgrowth—PC12 cells and c-src^{K295R}-expressing PC12 cells [srcDN2 cells; provided by Dr. S. Halegoua (State University of New York at Stony Brook, New York)] (*24*) were maintained in DMEM supplemented with 5% horse serum and 5% FCS. PC12 cells expressing RasN17 [M-M17-26 cells; provided by Dr. G.M. Cooper (Dana-Farber Cancer Institute, Harvard Medical School, MA)] (*25*) were maintained in DMEM supplemented with 5% horse serum, 5% FCS, and 400 μg/ml G418. The cells were passaged every 3-4 days and maintained at 37°C in 10% CO_2 under humidified air.

PC12 cells, srcDN2 cells, and RasN17 cells were seeded in the growth medium at 4.5×10^3 cells/cm^2 in collagen type I (Becton Dickinson)-coated 24 well culture plates and allowed to grow for 24 h. NGF, p15, and CPT-cAMP were added, and neurite outgrowth was measured after 48 h. For depolarization-stimuli, cells were depolarized by the addition of a depolarization solution to a final concentration of 40 mM. To reduce the extracellular Ca^{2+} concentration, PC12 cells were preloaded with Ca^{2+} chelator BAPTA/AM (20 μM; Research Biochemicals International) for 90 min and then washed with Ca^{2+}-free DMEM (Gibco; Cat. No. 21068-028) three times. The supplementation of Ca^{2+} was achieved by the addition of a $CaCl_2$ solution to a final concentration of 2 mM. For quantification of neurite outgrowth, five random photographs were taken per well, cells bearing processes longer than the cell diameter being considered positive.

Purification of p15—The fungal strain that produced p15 was precultured in flasks containing 100 ml of seed medium (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°C and 120 rpm for 1 week. Then the cells were reseeded into 5-liter flasks containing 1.5 liter of production medium (3% soluble starch, 1% peanut powder, 1% soybean meal, 1% KH_2PO_4, 1% NaH_2PO_4) and then incubated on a rotary shaker at 25°C and 120 rpm for a further 1 week. From 9 liters of culture broth, mycelia were collected by centrifugation and extracted with 70% (v/v) acetone. After centrifugation at 7,000 rpm for 30 min, the supernatant was collected and acetone was removed *in vacuo*. Then the extract was treated with equal volumes of diethylether, followed by ethylacetate. The aqueous phase was collected, and the ethylacetate and diethylether were removed *in vacuo*. A one-tenth volume of buffer A (500 mM Tris-HCl, pH 8.0) was added to the extract. Approximately one-twentieth of the 9-liter culture extract was applied to a DEAE-Toyopearl column (2 cm × 15 cm) equilibrated with 10-fold diluted buffer A and then eluted with a linear gradient of 0-0.5 M NaCl. The eluted fractions (5 ml/fraction) were analyzed for neurite outgrowth activity. p15 was eluted at around 0.1 M NaCl.

Carboxymethylation of p15—p15 (1 nmol) was dissolved in 1.5 mM Tris-HCl (pH 8.5) containing 7 M guanidine-HCl and 65 mM dithiothreitol (1 mg), and then incubated at 37°C for 1 h. Iodoacetic acid (2.5 mg) was added, followed by incubation in the dark for 1 h. After incubation, carboxymethylated p15 was recovered by reverse-phase (RP)-HPLC on an Aquapore RP-300 column.

Digestion with Proteases and Chemical Cleavage—Carboxymethylated p15 was used for digestion with proteases and chemical cleavage unless stated otherwise. Proteolytic digestions with AP-I (*26*) and endoproteinase Asp-N were carried out at 37°C in 100 mM Tris-HCl (pH 9.0) containing 10 mM EDTA and 2 M urea (total volume, 100 μl) for 18 h at an enzyme to substrate ratio of 1:50 to 1:200 (mol/mol). Methionyl bond was cleaved according to the method of Gross (*27*) with 1% (w/v) cyanogen bromide (CNBr) in 70% formic acid. Tryptophanyl bond was cleaved with BNPS-skatole in acetic acid, as described by Omenn *et al.* (*28*). To determine the carboxy-terminal fragment, the peptides digested with AP-I were applied to an anhydrotrypsin-agarose column (100 μl) which had been previously equilibrated with 50 mM sodium acetate (pH 5.0) and washed with 5 volumes of 50 mM sodium acetate (pH 5.0). The total flow through fraction (600 μl) was dried and then dissolved in 80% acetonitrile. The recovered fragments were separated by RP-HPLC on a Mightsyl RP-13 column. To determine the disulfide bond pairs, p15 and carboxymethylated p15 were dissolved in 8 M urea. The mixture was digested with AP-I at an enzyme to substrate ratio of 1:50 (mol/mol) in 100 mM Tris-HCl (pH 9.0) containing 10 mM EDTA and 2 M urea (total volume, 100 μl) at 37°C for 18 h.

Separation of Peptides—Peptides were applied to a column which had been previously equilibrated with 0.09% trifluoroacetic acid (TFA) and eluted with a linear gradient of acetonitrile containing 0.075% TFA, using a Hewlett-Packard Model 1090 M liquid chromatograph. A flow rate

of 0.2 or 0.5 ml/min was used for a column of 2.1 or 4.6 mm diameter, respectively. The effluent was monitored at 215 and 275 nm, using a Hewlett-Packard Model 1040M diode array detector.

Mass Spectrometry—Mass spectrometry analyses of intact p15 and selected peptides were performed by MALDI-TOF MS on REFLEX (Bruker-Franzen Analytik, Bremen, Germany) with a-cyano-4-hydroxycinamic acid or sinapinic acid as a matrix.

Nomenclature of Peptides—Peptides are designated by a serial number prefixed with a letter. The letter indicates the type of digestion: D, endoproteinase Asp-N; K, *Acromobacter* protease I; NK, non-reduced digestion of AP-I; M, CNBr; W, BNPS-skatole.

Gel Electrophoresis and Immunoblotting with Anti-Neurofilament M—SDS-PAGE was performed on 7.5% acrylamide gels under reducing conditions. The gels were stained with Coomassie Brilliant Blue. For Western blotting, PC12 cells grown on collagen type I-coated 60 mm culture dishes were treated with various concentrations of p15 for 48 h. The cells were washed three times with DMEM and then incubated in 0.5 ml per dish of lysis buffer [20 mM HEPES (pH 7.2), 10% glycerol, 1% NP-40, 1 mM NaF, 0.5 mM EGTA, 0.5 mM PMSF, and 1 μg/ml leupeptin] for 15 min at 4°C. The lysates were removed from the dishes after fully removing any remaining cellular debris with a cell scraper and then incubated in a microtube on ice for an additional 10 min. Then the lysates were clarified by centrifugation at 12,000 $\times g$ for 20 min and the supernatants were collected. Equal amounts of proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes using a semi-dry blotter (Nohon Eido, Tokyo). The blotted membranes were blocked with 5% skim milk in TBS (20 mM Tris-HCl, pH 7.4, 150 mM NaCl). The membranes were then probed with rabbit polyclonal antiserum to neurofilament M (1:1,000 dilution; AFFINITI Research Products Ltd.) for 1 h, followed by a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG secondary antibody (1:2,000 dilution; Vector), and developed using SuperSignal substrate (Pierce). Protein concentration was measured by the method of Bradford (*29*), using bovine serum albumin as a standard.

MAPK Activity—In-gel kinase assays were performed as previously described (*30*). Cell lysates (5-10 μg protein) were run on SDS-polyacrylamide gels containing myelin basic protein (MBP) as a substrate. Following electrophoresis, SDS was removed from the gels, the protein was denatured and renatured, and kinase assays were carried out by incubating the gel in buffer containing [γ-^{32}P]ATP (NEN NEG502; 10 Ci/mmol; 2 mCi/ml). The gels were washed and dried, and then the incorporated radioactivity was quantified using an image analyzer.

Immunoprecipitation and Src Kinase Activity—Activated and control PC12 cells were immediately solubilized with an equal volume of lysis buffer [0.1% SDS, 0.5% deoxycholate, 1% NP-40, 150 mM NaCl, 1 mM orthovanadate, 1 mM NaF, 50 mM Tris-HCl (pH 8.0), 0.5 mM EGTA, 1 mM PMSF, and 1 μg/μl leupeptin] for 15 min, and the resulting lysates were centrifuged at 12,000 $\times g$ for 20 min at 4°C. Src immune complexes were isolated using 10 μl of rabbit polyclonal antiserum to Src (OP07; CALBIOCHEM) per sample and 50 μl of protein A-Sepharose. The immunoprecipitates were washed twice with lysis buffer and then the Src kinase activities of each sample was assayed using a Universal Tyrosine Kinase Assay Kit (Takara, MK410) as described in the protocol.

RESULTS

Induction of Neurite Outgrowth from PC12 Cells by p15—In previous studies, we used polyethyleneimine (PEI)-coated culture plates to assess the neurite outgrowth from PC12 cells. A saturating dose of nerve growth factor (NGF), 50 ng/ml, induced neuritogenesis in only 10% of the cells after 48 h culture on PEI-coated plates, and p15-treatment alone failed to induce neurites at all. When cells were treated with NGF together with p15, neurite outgrowth was markedly enhanced (*23*). In the present study, we examined the effect of p15 on neuritogenesis in PC12 cells on collagen-coated culture plates, since it has been reported that collagen is the most effective purified extracellular matrix component supporting PC12 cell adhesion and NGF-induced neurite outgrowth (*31*). We found that p15 alone was capable of inducing neurites on collagen-coated plates; as shown in Fig. 1A, a saturating dose of p15 (1 nM) induced significant neuritogenesis in PC12 cells. The induction of neurites was dose-dependent at concentrations of 0.001-1 nM of p15 (Fig. 1B). Neurite outgrowth induced by p15 was faster than that by NGF; after 6 h of p15 treatment, small neurites were induced from PC12 cells, while on NGF treatment 24 h was required before detectable neurites were formed. Maximal neurite outgrowth was observed after 24-48 h of p15 treatment, the extent being comparable to that observed after 48-h treatment with NGF. Morphologically, p15-induced neurites were slightly thinner than NGF-induced ones. To determine whether or not the processes induced by p15 were authentic neurites, we examined the expression of neurofilament (NF)-M using an ELISA technique (data not shown) and Western blot analysis (Fig. 1C). NF-M is expressed specifically in neurons and increases in PC12 cells on treatment with neurite outgrowth stimulatory agents such as NGF (*2, 32, 33*). The protein level of NF-M markedly increased in p15-treated PC12 cells in a dose-dependent manner. These results demonstrate that the processes induced in p15-treated PC12 cells were actually neurites, indicating that PC12 cells undergo neuronal differentiation upon stimulation with p15.

Determination of the Complete Amino Acid Sequence of p15—p15 was purified from a culture extract of the producing fungal strain as described under "EXPERIMENTAL PROCEDURES," and its amino acid sequence was determined. Figure 2A shows the overall strategy used for determination of the amino acid sequence of p15. Almost all of the sequences were established by automated Edman degradation of intact p15, fragment W1 derived on BNPS-skatole cleavage and fragment M2 derived on CNBr cleavage. The sequences of the remainders and overlaps covering the major fragments were determined based on the sequence data for peptides K1 through K5 obtained on digestion with *Acromobacter* protease I (*26*) as well as the molecular masses of the peptides derived on digestion with endoproteinase Asp-N. The molecular weights of p15 and selected peptides were determined by MALDI-TOF MS to confirm the sequences obtained on automated Edman degradation. The molecular masses of p15 and reduced

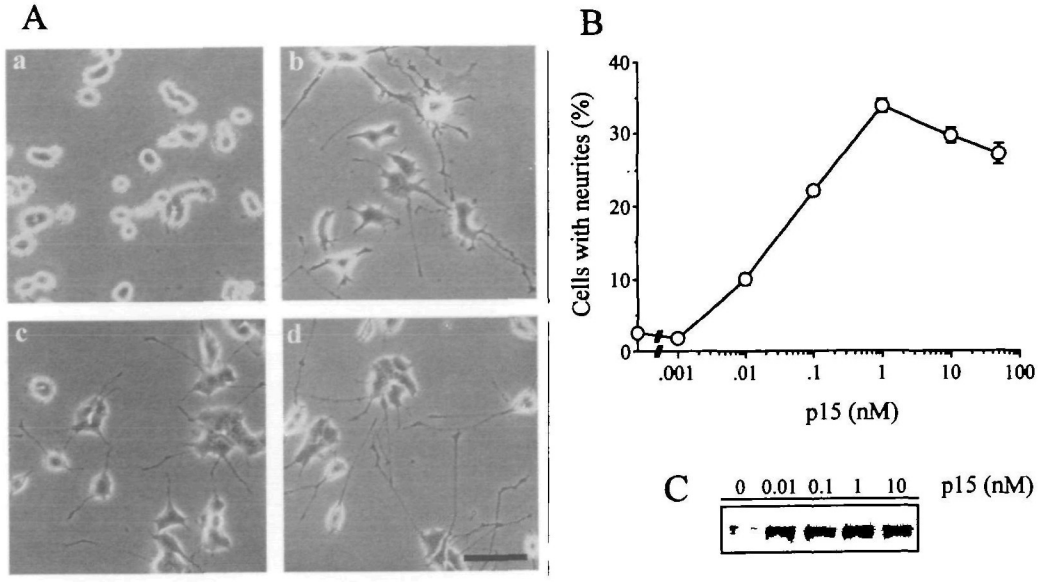


Fig. 1. **Induction of neurite outgrowth of PC12 cells by p15.** A: Approximately 1.4×10^3 cells were seeded into 24-well culture dishes, incubated for 24 h, and then incubated for 48 h with the experimental reagents. a, untreated control; b, NGF (50 ng/ml); c, p15 (0.1 nM); d, p15 (1 nM). Bar, 100 μm. B: PC12 cells were treated with various concentrations of p15 for 2 days. A total of over 500 cells was counted by using 4 wells per data point. Each value represents the mean ± SE of three independent experiments. C: PC12 cells were treated with various concentrations of p15 for 2 days. Cytoskeletal proteins were extracted and solubilized, and the immunoreactivity of NF-M protein was examined by Western blot analysis after SDS-PAGE separation.

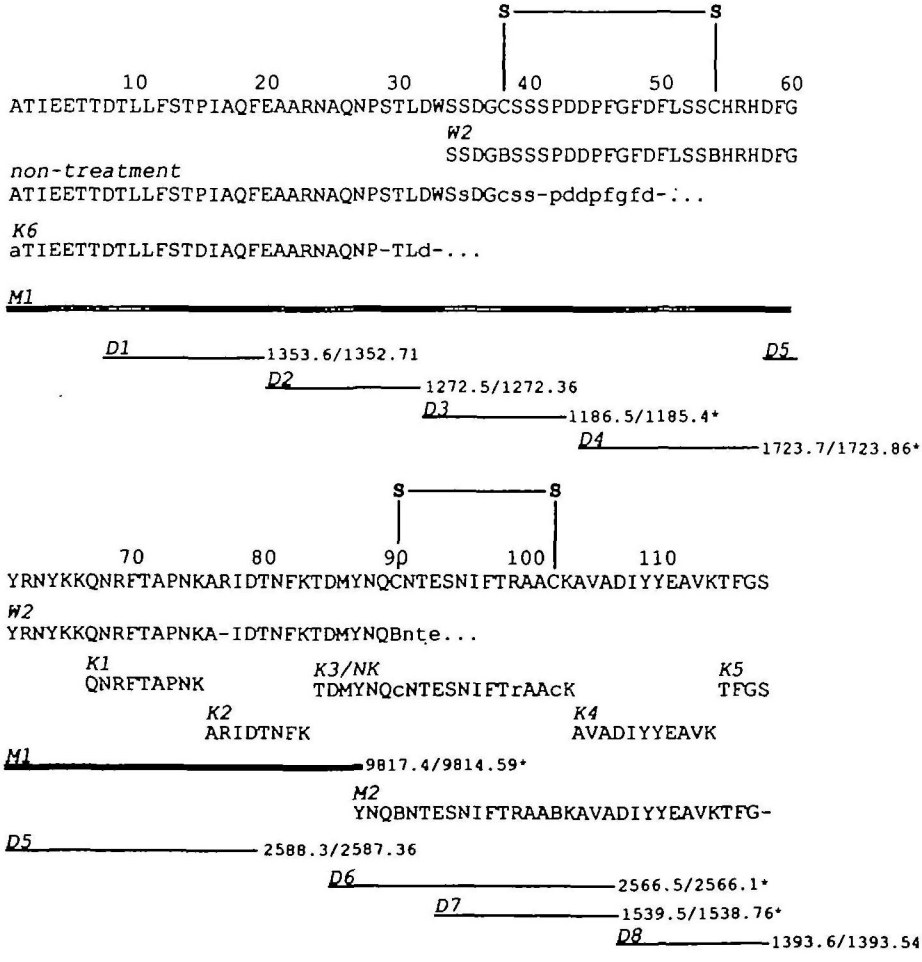


Fig. 2. **Summary proof of the amino acid sequence of p15.** The sequences determined by Edman degradation are given in a one-letter code below the summary sequence (top lines). Prefixes K, M, D, and W denote the agent used for the generation of the peptide, *i.e.* AP-I, CNBr, Asp-N, and BNPS-skatole, respectively. The peptides analyzed by mass spectrometry are indicated by double lines along with the observed/calculated protonated mass values. The peptide sequences shown in uppercase letters were proven by Edman degradation, and those in lowercase letters were tentatively identified. Those not identified are shown by dashes. B indicates carboxymethylcysteine. The molecular mass values with asterisks (*) were calculated as the carboxymethylated values.

carboxymethylated p15 were determined to be 13,304 and 13,539, respectively. The difference of 235 in the mass values indicates there are four cysteine residues in p15, all of which are involved in the formation of two disulfide bridge (the mass of a carboxymethyl group is 59). This result was consistent with the amino acid composition (data not shown). To determine the locations of the two disulfide bonds, p15 was digested with AP-I under non-reducing conditions. The chromatographic pattern was similar to that of the reduced digest. As shown in Fig. 2A, two cysteine residues are contained in fragment K3. Therefore, molecular mass analysis of fragment NK, which corresponds to K3 in the reduced digest, was performed. It was demonstrated that the molecular weights of fragment K3 and NK were 2,311 and 2,309, respectively. These results indicate that one of the disulfide bonds links Cys^{90} to Cys^{102} within fragment K3. Overall, we concluded that there are two disulfide bonds in intact p15, which link Cys^{38} to Cys^{54} and Cys^{90} to Cys^{102}.

Inhibition of the Neurite Outgrowth-Inducing Effect of p15 on Treatment with a Ca^{2+} Channel Blocker—Previously, we reported that p15 enhances NGF-induced neurite outgrowth from PC12 cells (*23*). We therefore examined if p15 exerts its effect through augmentation of the activity of TrkA tyrosine kinase. It has been reported that NGF-induced neurite outgrowth from PC12 cells is dramatically suppressed by treatment with K252a, a potent inhibitor of TrkA tyrosine kinase activity (*34*). This finding was essentially reproduced in our experiments; in the presence of 50 ng/ml NGF, 35.3±3.9% of cells had neurites longer than the cell diameter, while 11.6±0.3% of NGF- and K252a (50 nM)-treated cells had neurites. In contrast, K252a had little effect on p15-induced neurite outgrowth (Fig. 3A). This suggests that p15 does not directly affect the activity of TrkA, but rather affects distinct signaling pathway(s) to induce neurite outgrowth.

Solem *et al.* reported that depolarization stimuli synergize with subthreshold activation of NGF receptors to induce neurite outgrowth (*35*). We then compared the effects of depolarization and p15 treatment on neuritogenesis. Depolarization was achieved by adding a KCl solution to the medium. As reported, PC12 cells underwent the maximal neurite outgrowth in response to 40–50 mM KCl in our cultures. The ability of depolarization to induce neurite outgrowth was enhanced by 100 nM BayK 8644, an agent that remarkably increases the mean open time of L-type Ca^{2+} channels (data not shown). The extension of neurites induced by depolarization (40 mM KCl) was almost completely blocked by the L-type Ca^{2+} channel

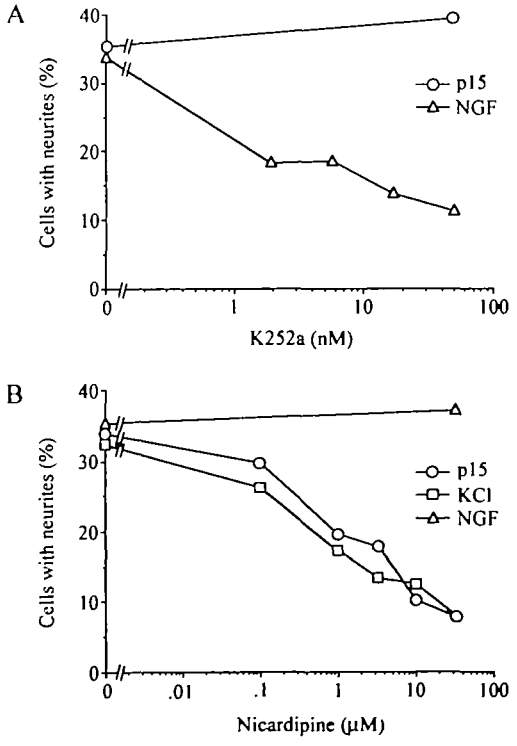


Fig. 3. **Effects of K252a and nicardipine on neurite outgrowth.** PC12 cells were stimulated with p15 (1 nM), KCl (40 mM), or NGF (50 ng/ml) for 48 h in the presence of the indicated concentrations of K252a (A), or nicardipine (B). The results shown are percentages of the total numbers of cells. The experiments were repeated at least three times with no significant difference.

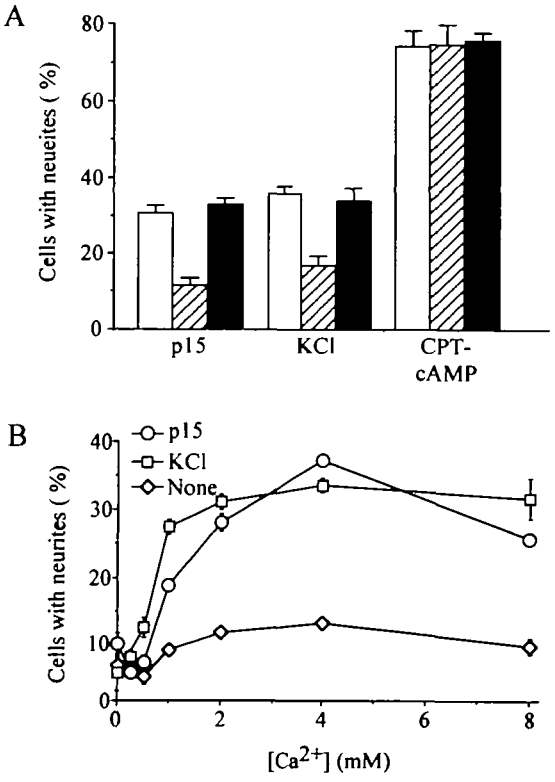


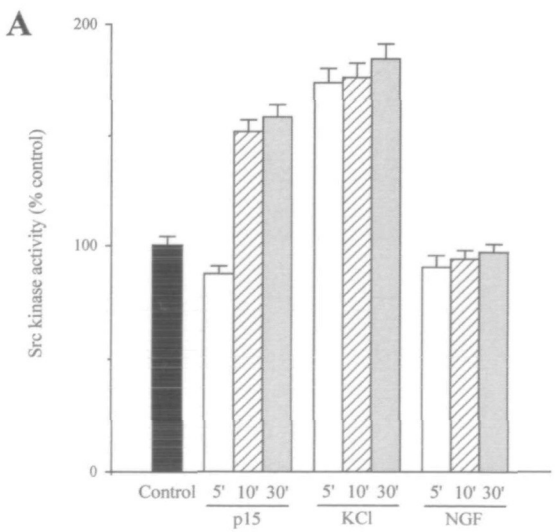
Fig. 4. **p15-induced neurite outgrowth is abolished by reducing extracellular Ca^{2+}.** A: After preloading PC12 cells with Ca^{2+} chelator BAPTA/AM (20 μM) for 90 min at 37°C, the culture media were replaced with Ca^{2+}-free DMEM containing p15 (1 nM), KCl (40 mM), or CPT-cAMP (100 μM) for 48 h. The results shown are percentages of the total numbers of cells. Open bars, control media; striped bars, Ca^{2+}-free media; solid bars, 2 mM Ca^{2+} was added to the Ca^{2+}-free media. The neurite-inducing activity of p15 was abolished by reducing extracellular Ca^{2+} (striped bar), but was recovered on re-addition of 2 mM Ca^{2+} (solid bar). The experiments were repeated at least three times with no significant difference. B: After preloading PC12 cells with Ca^{2+} chelator BAPTA/AM (20 μM) for 90 min at 37°C, the culture media were replaced with DMEM containing various concentrations of Ca^{2+} for 48 h. The results are the percentages of the total numbers of cell. The experiments were repeated at least three times with no significant difference.

blocker nicardipine (Fig. 3B). This indicates that depolarization-induced neurite outgrowth in PC12 cells is dependent on the influx of extracellular Ca^{2+} through L-type Ca^{2+} channels. Similarly, p15-induced neurite outgrowth was significantly inhibited by treatment with nicardipine. The inhibitory effect of nicardipine on depolarization- or p15-induced neurite growth was dose-dependent at concentrations of 0.1–33 μM. In contrast, nicardipine exhibited no effect on NGF-induced neurite outgrowth. These results suggest that the influx of extracellular Ca^{2+} through L-type Ca^{2+} channels is required for p15-induced neurite outgrowth.

To show unequivocally that p15-induced neurite outgrowth was indeed dependent on the presence of extracellular Ca^{2+}, PC12 cells were preloaded with BAPTA/AM, and then cultured in Ca^{2+}-free DMEM in the presence of p15. As shown in Fig. 4A, the induction of neurite outgrowth on treatment with p15 was significantly suppressed, but not when Ca^{2+} was readded to Ca^{2+}-free DMEM at 2 mM. Identical results were obtained for depolarization-induced neurite outgrowth. In contrast, the induction of neurite outgrowth by treatment with cAMP (CPT-cAMP) was not inhibited, although NGF-induced neurite outgrowth was slightly affected (data not shown). Basal neurite outgrowth was not affected by reducing the extracellular Ca^{2+} level to 0.25 mM or increasing it to 8 mM, but p15- or depolarization-induced neurite outgrowth was absolutely dependent on the extracellular Ca^{2+} concentration being >0.25 mM, the response being saturated at 2 to 4 mM (Fig. 4B). Taken together, these results strongly suggest that p15 induces neurite outgrowth by potentiating the influx of extracellular Ca^{2+} *via* L-type Ca^{2+} channels.

p15-Induced Neurite Outgrowth Depends on Src and Ras Activities—It was recently reported that depolarization-induced neurite outgrowth was inhibited by the expression of a dominant negative form of Src, chicken c-src^{K295R} (*20*). We next investigated the effect of p15 on Src kinase activity (Fig. 5A). Cells were treated with p15, KCl, or NGF for 5, 10, or 30 min. Then cell lysates were prepared and Src kinase activities in the immunoprecipitates obtained with anti-Src antibodies were assayed by means of an ELISA technique using a synthetic substrate. Depolarization induced an approximately 1.7-fold increase in Src kinase activity as early as 5 min after the addition of 40 mM KCl. There was no apparent increase in kinase activity at 5 min after p15 treatment, but longer treatment enhanced Src kinase activity to level that was similar to that on depolarization treatment. In contrast, NGF did not induce the increase in Src kinase activity. Thus, it appeared that p15 induces neurite outgrowth in PC12 cells *via* a mechanism that is dependent on the Src kinase activity.

To determine whether or not Src activity is required for the p15-induced neurite outgrowth, we next investigated the neurite-inducing activity of p15 toward the PC12 subline (srcDN2) that stably expresses the dominant-interfering form of Src, c-src^{K296R} (*20, 24*). As reported, srcDN2 cells were defective in depolarization-induced neurite outgrowth, while treatment with NGF caused neurite



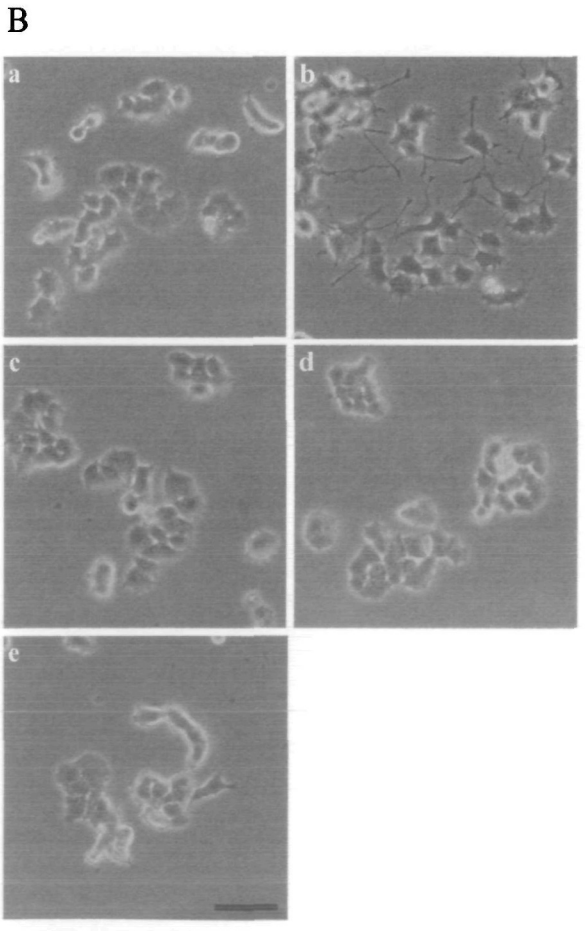


Fig. 5. **The neurite-inducing activity of p15 is dependent on Src kinase activity.** A: Cells were treated with p15 (1 nM), KCl (40 mM), or NGF (50 ng/ml) for 5 to 30 min, and then the Src kinase activities in the immunoprecipitates obtained with anti-Src antibodies were assayed by means of an ELISA technique using a synthetic substrate. The results are presented as the fold increase in Src kinase activity compared to in untreated cells (solid bar). The experiments were repeated at least twice with no significant difference. B: PC12 cells expressing dominant negative c-src^{K295R} (srcDN2 cells) were treated with the experimental reagents. a, p15 (1 nM); b, NGF (50 ng/ml); c, KCl (40 mM); d, KCl plus p15; e, KCl plus BayK8644 (100 nM). The experiments were repeated at least three times with no significant difference. Bar, 100 μm.

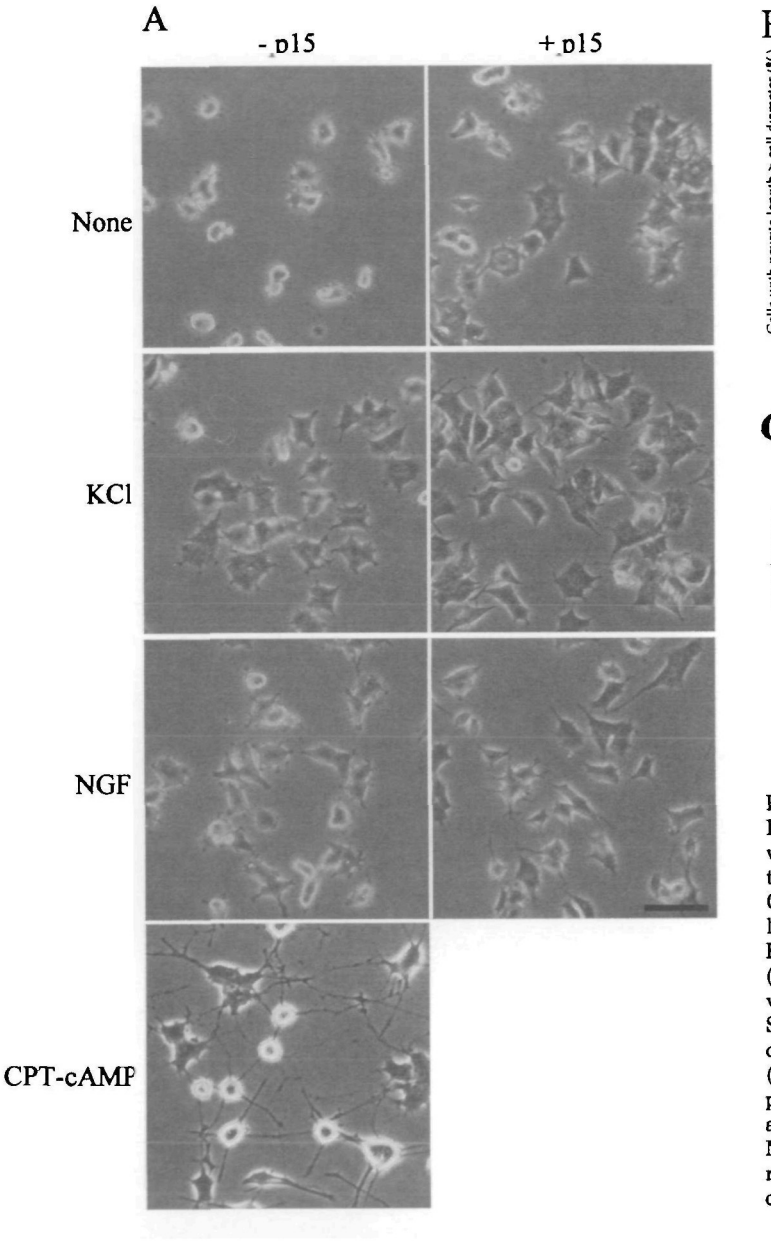


Fig. 6. **Activation of the Ras-MAPK cascade by p15.** A: PC12 cells expressing dominant-negative RasN17 (RasN17) were incubated in the presence or absence of p15 (1 nM) together with or without KCl (40 mM), NGF (50 ng/ml), or CPT-cAMP (100 μM). The experiments were repeated at least three times with no significant difference. Bar, 100 μm. B: Wild type PC12 cells were treated with p15 (1 nM), KCl (40 mM), or NGF (50 ng/ml) for 2 days in the presence of various doses of PD98059. Each value represents the mean ± SE of three independent experiments. C: Wild type PC12 cells were incubated with p15 (10 nM), KCl (40 mM), or NGF (50 ng/ml) for the indicated times. Cell lysates were electrophoresed on a 10% SDS-polyacrylamide gel and then MAPK activity was determined by means of an in gel-kinase assay. MAPK activity was quantified using an image analyzer. The results are presented as the fold increase in MAPK activity compared to untreated cells.

outgrowth (Fig. 5B). No obvious extension of neurites could be observed with p15-treatment. Collectively, these results demonstrate that neurite outgrowth induced by p15 is dependent on Src kinase activity.

Since the activation of MAPK, that is located downstream of Src, has been shown to play an important role in neuronal differentiation in PC12 cells, we examined if the activation of Ras and MAPK is required for p15-induced neurite outgrowth. Dominant inhibitory RasN17-expressing PC12 cells (RasN17) were treated with p15 in combination with NGF or depolarization (Fig. 6A). With any of these treatments, marked outgrowth of neurites was not observed, although treatment with p15 plus NGF or 40 mM KCl gave short spikes, and CPT-cAMP induced neurites. An MEK inhibitor, PD98059, also caused concentration-dependent inhibition of p15-induced neurite outgrowth, although it was not completely blocked (Fig. 6B). We next examined MAPK activities by means of an in-gel kinase assay using myelin basic protein as the substrate (Fig. 6C). NGF had stimulated MAPK activity at 5 min after the addition of NGF, and then the activity decreased with time. Similarly, depolarization or p15 transiently stimulated the MAPK activities, although to a much lower extent than NGF. These results indicate that p15 induces neurite outgrowth from PC12 cells, at least in part, through activation of the Ras-MAPK cascade.

DISCUSSION

Neuritogenesis of rat pheochromocytoma PC12 cells has often been used as a model for studying the biochemical mechanism of neuronal differentiation. Using this cell line, various compounds have been found to induce neurite outgrowth. We previously reported the identification of a novel fungal protein, p15, which enhances neurite outgrowth from NGF-treated PC12 cells (*23*). Even in the

presence of the maximal dose of NGF, p15 markedly enhanced NGF-induced neurite outgrowth, although p15 alone failed to induce neurites in the absence of NGF. In the present study, however, we found that p15 did induce neurites in the absence of NGF. The difference between the previous and current experiments is in the coating material for the culture plates. All our previous experiments involved PEI-coated substrata, while in this study we used collagen as the coating material. We found that on PEI-coated plates a saturating dose of NGF had induced neuritogenesis in only 10% of the cells after 48 h culture. Furthermore, depolarization-induced neurite outgrowth was not observed on PEI. It has been reported that collagen is the most effective purified extracellular matrix component supporting PC12 cell adhesion and NGF-induced neurite outgrowth (*31*). In this sense, p15-induced neurite outgrowth might require simultaneous activation of the integrin-mediated cell adhesion signaling pathway.

The complete amino acid sequence of p15 was determined by sequencing analyses of fragments obtained on chemical and enzymatic degradation of purified p15. Since the first amino acid of p15 determined was alanine, it is likely that p15 was processed from a precursor with an N-terminal signal sequence. In support of this speculation, the amino acid sequence of p15 exhibits significant homology to the mature region of a hypothetical secretory protein, CAB38593.1, of *Streptomyces coelicolor*, which has a potential N-terminal signal sequence (accession number AL035654). Because the locations of cysteine residues were conserved between p15 and CAB38593.1, it is likely that CAB38593.1 has similar activity to p15, which is now being examined.

We found that neurite outgrowth induced by p15 was inhibited by an L-type Ca^{2+} channel blocker, nicardipine. Since depolarization-induced Ca^{2+} influx activates Src kinase, we examined if activation of Src is involved in the neurite-inducing signal elicited by p15. Immunoprecipitates of p15-treated cell extracts with anti-src antibodies exhibited increased kinase activity. Furthermore, p15 and depolarizing stimuli failed to induce neurite outgrowth in srcDN2 cells, a subline of PC12 expressing the kinase-defective form of Src. Thus, the neurite-inducing activity of p15 requires Src kinase activity.

The Ras/MAPK signaling pathway also plays an important role in neuronal differentiation in PC12 cells (*12, 13*). Recently, it was shown that depolarization-induced Ca^{2+} influx can also activate this signaling pathway (*19-21*). Rusanescu *et al.* found that depolarization-induced Ca^{2+} influx into cells activates the Ras/MAPK signaling pathway *via* Src. Several lines of evidence suggest MAPK activation is involved in p15-induced differentiation of PC12. First, for the dominant inhibitory mutant RasN17 (RasN17 cells), marked outgrowth of neurites was not observed, although treatment with p15 plus NGF or depolarization gave short spikes. Second, an MEK inhibitor, PD98059, caused concentration-dependent inhibition of p15-induced neurite outgrowth (data not shown). Third, transient activation of MAPK activity was seen in p15-treated PC12 cells. These results do not necessarily preclude the possibility that p15 activates additional pathway(s) to the Ras/MAPK cascade. Indeed, inhibition of p15-induced neuritogenesis by PD98059 was not complete, and the MAPK activation by p15 was significantly low compared with that by NGF. Nonetheless, the results obtained in this study demonstrate that p15- or depolarization-induced neurite outgrowth depends on, at least partially, the Ras/MAPK signaling pathway.

Recently, many studies suggested that signaling pathway(s) which induce neuronal differentiation of PC12 cells in the absence of MAPK activation exist (*33, 36, 37*). p15-induced neurite outgrowth was inhibited by the Ca^{2+}/calmodulin kinase II inhibitor KN-93 (data not shown), thus raising the possibility of Ca^{2+}/calmodulin kinase involvement in the p15-induced signaling pathway. Further work is needed to identify the signaling pathway for p15-induced neurite outgrowth.

It remains unclear how p15 acts on the activation of the Ca^{2+} signaling pathway. Since p15 was purified from an extract of mycelia with 70% (v/v) acetone, one likely characteristic feature of p15 is a hydrophobic nature. This characteristic may be relevant to the unique activity of p15, because, in general, hydrophobic molecules are membrane permeable. α-Latrotoxin, the venom of the black widow spider, is inserted into membranes and serves as a non-selective cation channel (*38, 39*). This activity potentially explains the ability of α-latrotoxin to cause neurotransmitter release in the presence of Ca^{2+}, since α-latrotoxin could function as a Ca^{2+} ionophore. Alternatively, interaction with molecule(s) on the plasma membrane may be a trigger of neurite-inducing activity of p15. The neurite-inducing activity of p15 is saturated at 1 nM, suggesting that p15 exerts its effect after binding its high affinity receptor. The B subunit of cholera toxin (CTB) stimulated neuritogenesis in N18 cells and induced neurite branching in primary chick dorsal root ganglion (DRG) neurons (*40, 41*). Carlson *et al.* reported that CTB bound to ganglioside GM1 in the outer leaflet of cell membranes with high affinity and increased Ca^{2+} influx through L-type Ca^{2+} channels (*42*). To determine whether or not p15 could bind to gangliosides, the binding of avidin-conjugated p15 to gangliosides was examined by the ELISA technique. As reported (*43*), the binding of CTB to GM1 was highly detectable. However, we could not detect the binding of avidin-conjugated p15 to any gangliosides, such as GM1, GT1b, GM3, and GD1a (data not shown). This led us to suggest that p15 potentiates the L-type Ca^{2+} channels through a distinct pathway from that activated by CTB.

Two sets of indirect evidence that Ca^{2+} influx into cells mediates the neurite-inducing activity of p15 were obtained by showing that the depletion of extracellular Ca^{2+} or inhibition of L-type Ca^{2+} channels by a specific blocker inhibits neurite outgrowth from p15-treated PC12 cells. This suggests that the influx of extracellular Ca^{2+} through L-type Ca^{2+} channels is required for p15-induced neurite outgrowth. However, we failed to detect a change in the concentration of cytosolic Ca^{2+} on treatment with p15 using the Ca^{2+} indicator fura-2 (data not shown). In this regard, small or very local alterations in Ca^{2+} might have escaped detection. Many studies have suggested that Ca^{2+} influx through the plasma membrane influences neurite initiation, growth cone motility, and neuronal survival (*21, 44*). Kater and co-workers suggested that there is a narrow, optimal level of cytosolic free Ca^{2+} below or above which these processes are inhibited (*44, 45*). In rat cortical neurons, p15 inhibited neurite elongation and induced short, slender and less branched neurites. In contrast, p15 apparently did

not have any effect on neurites but promoted survival in the absence of NGF in rat DRG neurons (data not shown). Thus, the ability of p15 to modulate the differentiation and/or survival of neuronal cells may be related to the differences in the optimal level of intracellular free Ca^{2+}.

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