The Dual-Specificity CLK Kinase Induces Neuronal Differentiation of PC12 Cells

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CLK is a dual-specificity protein kinase capable of phosphorylating serine, threonine, and tyrosine residues. We have investigated the action of CLK by establishing stable PC12 cell lines capable of inducibly expressing CLK. Expression of CLK in stably transfected PC12 cells mimicked a number of nerve growth factor (NGF)-dependent events, including the morphological differentiation of these cells and the elaboration of neurites. Moreover, CLK expression enhanced the rate of NGF-mediated neurite outgrowth of these cells, indicating that CLK expression and NGF treatment activate similar signal transduction pathways. CLK expression, unlike NGF, was not able to promote PC12 cell survival in serum-free media, demonstrating that CLK only partially recapitulated the actions of NGF on these cells and that the biochemical pathways necessary for morphological differentiation can be stimulated without also stimulating those necessary for survival. Induction of CLK expression also resulted in the selective activation of protein kinases that are components of growth factor-stimulated signal transduction cascades, including ERK1, ERK2, pp90<sup>RSK</sup>, and S6PKII. Induction of CLK expression, however, did not stimulate pp70<sup>S6K</sup> or Fos kinase, two NGF-sensitive protein kinases. These data indicate that CLK action mediates the morphological differentiation of these cells through its capacity to independently stimulate signal transduction pathways normally employed by NGF.

The biological events leading to the acquisition of a neuronal phenotype have been extensively studied; however, it remains unclear how neuronal stem cell populations are instructed to withdraw from the cell cycle and selectively express neuronal cell-specific genes. The ability of nerve growth factor (NGF) to elicit such changes in the clonal rat pheochromocytoma (PC12) cell line has allowed the use of this model system for biochemical investigation of these events (26). Like neuronal stem cells, PC12 cells can either exit the cell cycle and terminally differentiate or continue to proliferate in a nondifferentiated state. NGF stimulation of PC12 cells initially prompts the cells to undergo a final round of mitosis, followed by withdrawal of the cells from the cell cycle. NGF treatment provokes the stimulation of multiple independent signaling pathways leading to phenotypic changes, including the development of synaptic vesicles, electrical excitability (22), and the elaboration of neurites (26).

NGF initiates its actions through binding to the trkA proto-oncogene. The trkA receptor possesses a tyrosine kinase domain whose activity is stimulated upon NGF binding (28, 36–38). Activation of the trkA receptor kinase leads to the tyrosine autophosphorylation of trkA and the subsequent association of SH2 domain-containing adapter and effector proteins with the receptor (53). Recent studies have defined a trkA-dependent proto-oncogene signaling cascade that results in the activation of p21<sup>ras</sup> (20, 39). Activation of p21<sup>ras</sup> results in the serial activation of serine/threonine kinases including the raf kinases, MEK1, the mitogen-activated protein (MAP) kinases (ERK1 and ERK2), and pp90<sup>RSK</sup> (20, 33, 49, 58, 60). The p21<sup>ras</sup>-dependent serine/threonine kinase cascades appear to be a major avenue responsible for transmission of intracellular signals from the membrane to the nucleus (21).

It has recently been recognized that several growth factor-stimulated kinases are members of the newly described class of dual-specificity kinases that have the capacity to phosphorylate serine, threonine, and tyrosine residues (44). Heretofore, protein kinases have been thought to be segregated into two distinct and nonoverlapping groups based on conserved amino acid motifs that were used to predict their ability to phosphorylate either serine/threonine residues or tyrosine residues (27, 44). On the basis of their primary amino acid sequences, the dual-specificity kinases are indistinguishable from serine/threonine kinases (44). However, their ability to phosphorylate tyrosine residues has forced a reevaluation of intracellular signaling pathways, since it was believed that only tyrosine kinases could be responsible for these modifications.

The first identification of a dual-specificity kinase came with the cloning of CLK (cdc2-like kinase) (6, 30, 35). CLK was isolated by screening expression libraries with antiphosphorytrosine antibodies and was subsequently shown to phosphorylate itself, as well as exogenous substrates, on both serine and tyrosine residues (6, 30). The highest levels of CLK expression were found in the brain (30). Moreover, differentiation of P19 embryonic carcinoma cells into a neural phenotype was found to be accompanied by the enhanced expression of CLK mRNA (30). However, these data provided few insights into the physiological function of CLK.

The recognition that the dual-specificity protein kinases are critical intermediates in the transduction of intracellular signals and their potential role during neuronal development (30) led us to investigate the capacity of the prototypic dual-specificity kinase, CLK, to intervene in NGF-mediated signaling events. We report here that expression of CLK in PC12 cells resulted in the selective activation of growth factor-dependent kinase cascades and resulted in the morphological differentiation of PC12 cells, indicating that CLK may function as a component of growth factor-dependent signal transduction cascades.

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MATERIALS AND METHODS

Cell culture and generation of CLK-expressing lines. PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum and 10% donor horse serum in an atmosphere of 10% CO2. PC-CLK lines were generated by electroporating PC12 cells with a pNeoNut construct containing the full-length CLK cDNA cloned downstream of the metallothionein-1 (MT-1) promoter (17). Stably transfected PC12 cell lines were established by electroporating PC12 cells with the pNeoNut vector alone (16). Electroporation of the cells was accomplished by resuspending the PC12 cells in electroporation buffer (100 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], 137 mM NaCl, 6 mM dextrose, 7 mM Na2PO4 [pH 7.4]) at a final density of 10^7 cells per ml. The resuspended cells (0.5 ml) were mixed with 20 μg of plasmid DNA at 4°C and then electroporated at 290 V and 960 μF with a Bio-Rad Gene Pulser. The cells were then immediately transferred into 10 ml of fresh medium. The medium was replaced with 50% PC12 cell conditioned medium containing 500 mg of G418 (GIBCO) per ml 48 h after electroporation. Following 3 weeks of selection with G418, 10 independent colonies were isolated and expanded.

RNA extraction and Northern (RNA) blot analysis. RNA was isolated by the method of Chomczynski and Sacchi (12), and the concentration of RNA was determined by reading the absorbance at 260 nm. Total RNA (30 μg) was separated by formaldehyde-agarose gel electrophoresis, blotted onto Nytran (Schleicher and Schuell), and probed with a radiolabeled CLK cDNA. Following hybridization, equal load and transfer of the RNA were verified by staining the blot for 15 min with 0.05% methylene blue in 0.5 M sodium acetate solution (pH 5.2).

Differentiation of PC12 cells. PC12 cells, PC-CLK4 cells, or PC12 cells transfected with the vector alone were plated onto a collagen substrate and treated with 5 μM ZnSO4 or with NGF (50 ng/ml) in serum-free media for various times. Three random fields from each of three independent wells were counted (approximately 200 cells per field) for the percentage of cells with neurites longer than 2 cell diameters.

Survival assays. PC12 cells, PC-CLK4 cells, or PC12 cells transfected with the vector alone were incubated in the presence or absence of 5 μM ZnSO4 for 4 days in serum-containing DMEM. The serum-containing medium was removed, and DMEM containing no serum was added to the cultures. The initial number of cells present was measured by counting cells within three identified fields in each of three independent wells (approximately 150 cells per field). The cells were then incubated for various times in the presence or absence of 5 μM ZnSO4 and NGF (50 ng/ml). After various periods, the cells remaining were counted to determine the fraction of viable phase-bright cells remaining in the wells.

Anion-exchange column chromatography. Wild-type PC12 cells, PC-CLK4, and PC12 cells transfected with the vector alone (10^7 cells) were incubated in the presence or absence of 5 μM ZnSO4 for 4 h and were then lysed in buffer A [20 mM Tris [pH 7.4], 1 mM EGTA [ethylene glycol-bis (β-aminoethyl ether)-N,N',N'-tetraacetic acid], 0.1 mM Na3VO4 containing 10 mM p-nitrophenyl phosphate. Following centrifugation at 100,000 × g for 35 min, the supernatants were applied to a MonoQ HR5/5 fast protein liquid chromatography column (Pharmacia) that had been preequilibrated with buffer A. The column was washed with 5 ml of buffer A and then developed with a linear 0 to 0.5 M NaCl gradient, and 1-ml fractions were collected, as previously described (34).

Protein kinase assays. Alternate fractions obtained from MonoQ chromatography were assayed for protein kinase activity by using either 1 μg of myelin basic protein (MBP) or 50 μM S6 peptide (RRRLSSLRRA) as the substrate. Kinase reactions were carried out in the presence of 10 mM MgCl2–2 mM MnCl2–[γ-32P]ATP (44 dpm/fmol) for 20 min at room temperature. MBP assays were stopped by the addition of concentrated Laemmli sample buffer, mixtures were boiled for 5 min, and the reaction products were separated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The bands corresponding to MBP were cut from the gel, and the incorporated radioactivity was measured by Cerenkov counting. S6 peptide assays were stopped by the addition of trichloroacetic acid to 3.5% and bovine serum albumin to 0.2 mg/ml, and mixtures were incubated for 15 min on ice as previously described (57). After centrifugation at 10,000 × g for 5 min, aliquots of the supernatants were spotted onto Whatman P-81 paper and washed three times for 5 min each time with 75 mM phosphoric acid (25). The incorporated radioactivity was measured by Cerenkov counting.

Immunoblotting. Fractions obtained from the MonoQ chromatography were trichloroacetic acid precipitated, and the proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred to an Immobilon P membrane as previously described (34). The blots were probed with ppp90 immunobodies provided by J. Avrich, ppp90 immunobodies purchased from UBI, the antiphosphotyrosine antibodies purchased from Signal Transduction Laboratories, and MAP kinase antisera purchased from Bio-Designs, Inc. The immunoreactive species were detected with horseradish peroxidase-conjugated secondary antibodies and visualized with enhanced chemiluminescence reagents obtained from Amersham.

RESULTS

Expression of CLK in PC12 cells. We established PC12 cell lines stably expressing CLK under the control of the MT-1 promoter. Two cell lines, PC-CLK4 and PC-CLK6, were selected for further characterization on the basis of their phenotype, response to Zn2+. Incubation of PC-CLK4 and PC-CLK6 cells in the presence of Zn2+ resulted in the transient induction of CLK mRNA (Fig. 1A). CLK mRNA levels were maximal within 3 h and then declined over the next several hours, but they were still detectable for at least 24 h (data not shown). The size of the Zn2+-inducible CLK mRNA (3.2 kb) is consistent with the size of the CLK cDNA fused to the untranslated regions of human growth hormone that flank the cloning site in pNeoNut. Staining of the blot with methylene blue verified that equal amounts of RNA were loaded and transferred (data not shown). CLK mRNA was not detected in Zn2+-treated or untreated wild-type PC12 cells, in cell lines transfected with the vector only, or in NGF-treated PC12 cells, indicating that these cells represent a novel host for CLK. The basis of the transient nature of CLK mRNA expression is unclear but is reminiscent of the behavior of v-mos expression when driven by a structurally similar heavy metal-inducible promoter (32).

CLK expression was also evaluated by detection of its enzymatic activity in Zn2+-treated PC-CLK4 cells. CLK activity was measured by the phosphorylation of MBP. PC-CLK4 cells, wild-type PC12 cells, and PC12 cells which were transfected with the pNeoNut vector alone were incubated for 4 h in the presence or absence of Zn2+. Lysates from these cells were applied to a MonoQ column to allow separation of CLK from the MAP kinases, which are the major MBP kinases in these cells (41). CLK has a predicted pI of 8.9 (30) and will not bind to this resin under the conditions used. We found that
Zn\textsuperscript{2+}-induced CLK differentiation of PC12 cells. Induction of CLK expression in PC-CLK4 cells resulted in the morphological differentiation of these cells, as evidenced by the elaboration of neurites (Fig. 2). The CLK-mediated neurite outgrowth of PC-CLK4 cells occurred more slowly than that induced by NGF (Fig. 3), and the neurites were shorter (Fig. 2) but were otherwise indistinguishable from neurites elicited by NGF. Importantly, Zn\textsuperscript{2+} treatment of the parental PC12 cells or cell lines transfected with the vector only did not result in neuronal differentiation. These cells, however, retained normal responsiveness to NGF (Fig. 3), indicating that the Zn\textsuperscript{2+}-induced neuronal differentiation seen in PC-CLK4 cells was not due to nonspecific cellular responses to potentially toxic heavy metals. PC-CLK6 cells also elaborated neurites in response to Zn\textsuperscript{2+} treatment, but to a lesser degree than did the PC-CLK4 cells (data not shown), which is reflected in the different levels of CLK mRNA induced by Zn\textsuperscript{2+} treatment (Fig. 1A). Because of their more robust response to Zn\textsuperscript{2+} treatment, PC-CLK4 cells were used in all subsequent studies.

CLK enhancement of NGF-mediated neurite outgrowth. The observation that CLK expression provoked the morphological differentiation of PC-CLK4 cells led us to question whether CLK expression could enhance subsequent NGF action, in a manner similar to that of NGF pretreatment (10). Pretreatment of PC-CLK4 cells with Zn\textsuperscript{2+} for 2 days resulted in an accelerated response to NGF (Fig. 3A). Importantly, Zn\textsuperscript{2+} treatment did not alter the rate of NGF-mediated neurite outgrowth in either the parental PC12 cell line or cells transfected with the vector alone (Fig. 3). These data demonstrate that the enhancement of neurite outgrowth was caused by the induction of CLK expression and not by nonspecific cellular responses to Zn\textsuperscript{2+}.

PC-CLK4 cells responded more quickly to NGF than did the cell lines transfected with the vector alone or the parental PC12 cell line. Moreover, Zn\textsuperscript{2+} treatment enhanced neurite outgrowth only from the PC-CLK4 cells (Fig. 3A). The accelerated response to NGF by untreated PC-CLK4 cells may be caused either by the selection of a more rapidly responding clone or, more likely, by low levels of CLK expressed by the notoriously leaky MT-1 promoter (10).

Effect of CLK expression on cell survival. PC12 cells normally require serum for survival and die within 96 h in its absence (51). NGF, however, has been shown to support the survival of these cells in serum-free media (51). We tested whether the induction of CLK expression was sufficient to sustain these cells upon serum withdrawal, mimicking another effect of NGF. PC-CLK4 cells were incubated for 4 days in the presence of 5 \mu M Zn\textsuperscript{2+} and were then incubated in the presence or absence of Zn\textsuperscript{2+} in serum-free media for various times, after which the number of surviving cells was determined. In contrast to CLK's capacity to augment the action of NGF on the differentiation of PC12 cells, Zn\textsuperscript{2+}-treated PC-CLK4 cells died as rapidly in the absence of serum as did the parental PC12 cells and the PC12 cells transfected with the vector alone (Fig. 4). These data demonstrate that CLK does not promote the serum-free survival of PC12 cells. Moreover, CLK served to stimulate the morphological differentiation of these cells without also promoting their survival. These data suggest that CLK cannot reproduce the entire range of NGF...
FIG. 2. CLK expression induces the neuronal differentiation of PC12 cells. PC-CLK4 cells (A and B) and PC12 cells (C and D) were incubated in the absence (A and C) or presence (B and D) of 5 μM Zn²⁺ for 7 days in serum-free media on a collagen substratum. Normal responsiveness to NGF was tested by treating PC12 cells (E) or PC-CLK4 cells (F) with NGF (50 ng/ml) for 7 days.
FIG. 3. CLK accelerates neurite outgrowth in PC12 cells. (A) PC-CLK4 cells and the parental PC12 cells were incubated in the presence or absence of 5 μM Zn²⁺ for 24 h. The incubation was continued for the indicated times in the presence or absence of 50 ng of NGF per ml. △, PC12; ○, PC12 plus Zn²⁺; □, PC12 plus NGF; ■, PC12 plus NGF plus Zn²⁺; (○), PC-CLK4; △, PC-CLK4 plus Zn²⁺; (○), PC-CLK4 plus NGF; □, PC-CLK4 plus NGF plus Zn²⁺. (B) The incubation of PC12 cells, and cells transfected with the vector only were incubated in the presence or absence of 5 μM Zn²⁺ for 48 h and then incubated in the presence or absence of 50 ng of NGF per ml for 48 h. The values for both panels represent the percentage of cells with neurites in three random fields and are from three independent trials (means ± standard errors of the mean [error bars]).

actions and acts within only a subset of the pathways normally stimulated by NGF.

CLK-activated signal transduction cascades. The CLK-induced morphological differentiation of PC12 cells suggested that CLK intervened in one or more NGF-dependent signaling cascades. We directly tested whether CLK expression resulted in the activation of protein kinases which are constituents of

FIG. 4. CLK does not promote the survival of PC12 cells. (A) PC-CLK4 cells or PC12 cells transfected with the vector alone were incubated in either the presence or absence of 5 μM Zn²⁺ for 4 days in serum-containing medium. The serum-containing medium was replaced with serum-free medium, and the number of adherent cells was determined. The percentage of surviving cells was determined at the indicated times. (B) The number of PC-CLK4 cells, wild-type PC12 cells, and PC12 cells transfected with the vector only were treated as described for panel A except the cells were then incubated in the presence or absence of 50 ng of NGF per ml for 4 days before the number of adherent cells was determined. The values for both panels represent the percentage of surviving cells (error bars indicate standard deviations; n = 3).

the NGF-dependent signaling cascades. PC12 cells, PC-CLK4 cells, or PC12 cells transfected with the vector alone were incubated for 4 h with 5 μM Zn²⁺, and the activities of MAP kinases, S6 peptide kinases, and Fos kinase were determined (57). CLK expression resulted in the stimulation of MAP kinase activities (Fig. 5B and C), reflecting the activation of ERK1 by 15-fold and ERK2 by 5-fold. Similarly, pp90RSK activity was stimulated sevenfold in the CLK-expressing cells (Fig. 5A and C). This latter observation is consistent with the capacity of the MAP kinases to directly phosphorylate and
activate pp90RSK (13, 15). Another S6 peptide kinase activity, termed S6PKII, was stimulated 18-fold upon induction of CLK (Fig. 5A and C). S6PKII represents a poorly characterized growth factor-responsive kinase previously reported in a number of studies (1, 24). The activation of these enzymes was a consequence of CLK expression, as Zn²⁺ treatment of the parental PC12 cells or cells transfected only with the vector did not stimulate MAP kinase activity and resulted in only a modest increase in pp90RSK activity and S6PKII (Fig. 5C).

The effect of CLK expression on NGF-dependent serine/threonine kinases was specific, as Zn²⁺ treatment of PC-CLK4 cells and the parental PC12 cells had only a small effect on the activities of pp⁷OS6K (Fig. 5A and C) and Fos kinase (data not shown), which were stimulated to almost the same degree. The response of cellular kinases to Zn²⁺ is most likely due to the ability of Zn²⁺ to effect both protein kinase activity (3) and phosphatase activity (9). These data confirm that CLK expression stimulated only a subset of the biochemical pathways utilized by NGF.

**DISCUSSION**

The existence of dual-specificity protein kinases was first suggested by the observation that the *Schizosaccharomyces pombe* wee1 gene product functionally inactivated cdc2-cyclin B complexes through the threonine and tyrosine phosphorylation of cdc2 (23, 47, 52). Subsequently, it has been shown that the dual-specificity kinases also play a central role in the initiation and maintenance of growth factor-mediated signal transduction. The activin type II receptor (46) and the MAP kinases (18) and their upstream activator, MEK1 (also termed MAP kinase kinase), are dual-specificity kinases (18, 19). MEK1 and the MAP kinases are crucial intermediates in growth factor-activated signal transduction cascades, as they have been shown to activate other kinases, such as pp⁹RSK (55) and MAPKAP kinase-2 (54), as well as to modulate transcription through the phosphorylation of a growing number of transcription factors (21, 31).

The present data provide direct evidence that expression of CLK mimicked a number of the actions of NGF, with respect to both the induction of morphological differentiation of PC12 cells and the capacity to activate protein kinases which are central elements of NGF-stimulated signal transduction pathways. The ability of CLK to stimulate the acquisition of a neuronal phenotype in PC12 cells may be of particular significance, given that the brain normally expresses CLK at much greater levels than other tissues (30). It is also of interest that the differentiation of P19 embryonal carcinoma cells to a neural phenotype is accompanied by a dramatic stimulation of CLK expression (30).

The CLK-induced morphological differentiation of PC12 cells is similar to that produced upon expression of the proto-oncogenes v-src (2), Ha-ras (5), v-ck (56), and shc (50) or oncogenic RAF (RAF-1) alleles (61). The induction of neuronal differentiation in PC12 cells is a specific response, as other ligands, such as insulin, phorbol esters, and epidermal growth factor (11), do not result in the differentiation of PC12 cells or augment the action of subsequent NGF treatment (10). CLK expression was able to enhance the action of NGF, indicating that CLK and NGF are likely to stimulate overlapping signal transduction cascades that generate similar biosynthetic events resulting in the morphological differentiation of PC12 cells.

The neurite outgrowth in response to CLK, however, was slower than the normal NGF response. This is likely due to the transient nature of the CLK mRNA expression. High levels of CLK expression were observed for only a few hours; however, expression of CLK was detected at significantly lower levels which were sufficient to elicit the long-term differentiation of the PC-CLK4 cells. CLK expression, in contrast to NGF treatment, was unable to promote the survival of PC12 cells in serum-free media. This finding is of particular significance.
because it reveals that the biochemical events necessary for the morphological differentiation of PC12 cells can be discriminated from those necessary to promote their survival. These data demonstrate that two physiologically distinct responses, differentiation and survival, are subjected to different regulatory controls, which are mediated by independent signaling pathways.

The mechanisms by which CLK activity is regulated and the positions within the signal transduction cascade where CLK acts are currently unknown. The trkA-mediated signals responsible for the activation of serine/threonine kinases and the acquisition of neuronal properties have been shown to be conveyed through pp60-src, p21^ras, and raf (20, 39). Recently, a number of groups have shown that the raf kinases directly associate with activated p21^ras (33, 45, 59) and can activate the MAP kinase activator, MEK1 (29, 40), leading to the serial activation of the MAP kinases and pp90^rsk. The ability of CLK to phosphorylate serine/threonine residues, as well as tyrosine residues, suggests that CLK could intervene at a number of places in the NGF-dependent signal transduction cascade. Most notably, activations of the MAP kinases (29, 40, 48) and their activator, MEK1 (5, 34), have been shown to be dependent on both serine/threonine and tyrosine phosphorylation. The activation of this cascade by a differentiation-specific stimulus, CLK, supports the importance of the MAP kinase cascade in mediating the morphological differentiation of PC12 cells (34, 58, 60).

Recently, Lange-Carter et al. (43) have described an alternate pathway leading to the activation of the MAP kinase cascade. In this pathway, MEK is activated by MEKK rather than by raf. This MEKK-dependent pathway is believed to be regulated by G-protein-linked receptors rather than by tyrosine kinase-linked receptors (43). The presence of this pathway, along with multiple MEK, and presumably MEKK, isoforms adds to the potential sites of CLK intervention in the intracellular signaling cascade.

An important feature of these studies is that a wild-type allele of CLK was employed in these experiments. The use of a nonactivated (i.e., oncogenic) allele makes it unlikely that the effects seen here are due to nonspecific interactions driven by a hyperactive kinase. The inability of CLK to activate Fos kinase or pp70^s6k is of particular significance, as it directly demonstrated that CLK selectively regulates the pathways through which serine/threonine kinases act to effect the biological actions of growth factors. pp70^s6k and Fos kinase are, however, components of the normal cellular responsiveness to NGF and other growth factors (42, 57). The ability of CLK to activate the MAP kinase cascade while failing to activate pp70^s6k is not too surprising, as these kinases are regulated by distinct signaling events (4, 7, 14). The inability of CLK expression to activate pp70^s6k and Fos kinase suggests that the activation of these protein kinases is not absolutely necessary for the morphological differentiation of PC12 cells.

CLK is not normally expressed in PC12 cells and therefore does not participate in NGF-mediated signal transduction. CLK’s responsiveness to differentiation stimuli in P19 cells (30) and CLK’s ability to differentiate PC12 cells suggest that CLK may normally act to promote and maintain the differentiated phenotype in the nervous system. The ability of CLK to participate in signal transduction pathways is consistent with the actions of its structural homologues, Dcd2 (6, 35) and the MAP kinases (8). The ability of CLK to selectively intervene within NGF signaling pathways suggests that CLK may also participate in growth factor signaling in other cell types.

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