Overexpression of Calbindin-D28K Induces Neurite Outgrowth in Dopaminergic Neuronal Cells via Activation of p38 MAPK

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An MN9D dopaminergic neuronal cell line overexpressing calbindin-D28K (MN9D/Calbindin) was established in order to investigate directly the potential role of calcium-binding protein in neuronal differentiation. Overexpression of calbindin-D28K in MN9D cells resulted in significant increases in the number of neurites, the length of primary neurites, and the total extent of neurites. This robust neurite outgrowth occurred without cessation of cell division. Analysis of immunoblots revealed that this morphological differentiation was accompanied by increased expression of such markers of maturation as the synaptosomal protein SNAP-25. During calbindin-D28K-evoked neurite outgrowth in MN9D cells, phosphorylation of p38 mitogen-activated protein kinase (MAPK) dramatically increased while the levels and extent of phosphorylation of such other MAPKs as c-Jun N-terminal kinase (JNK) or extracellular response kinase (ERK) were not altered. Consequently, calbindin-D28K-induced neurite outgrowth was largely abolished by treatment with a p38 inhibitor, PD 169316, while the level of SNAP-25 in MN9D/Calbindin cells was not altered by this treatment. These data support an idea that calbindin-D28K and its associated p38 signaling pathway play a role in dopaminergic neuronal differentiation.© 2001 Academic Press

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Calcium has been implicated in a variety of neuronal functions such as the control of neuronal excitability, transmission, differentiation, survival, and death (1). There are several loci that can regulate the level of intracellular calcium in neurons, which are involved consequently in various aspects of neuronal physiology and pathology. These loci include intracellular sites at which calcium is stored including mitochondria and endoplasmic reticulum, calcium pumps, and exchangers. In addition, such calcium-binding proteins as calbindin, parvalbumin and calretinin are distributed in several classes of neurons, and are believed to limit stimulated increases in intracellular free calcium concentration (2, 3).

Calbindin-D28K belongs to a family of proteins containing a calcium-binding domain designated as the EF-hand family (4). Until now, studies have focused on calbindin-D28K’s distribution and altered expression in various situations. In many regions of the central nervous system, the abundance of calbindin-D28K is particularly striking; interestingly, however, its overall distribution is specifically restricted (3). Other members of the EF-hand family of calcium binding proteins such as calmodulin and calpains have been extensively investigated with respect to their modulation of the physiological activities of neuronal cells. By contrast, the extent to which calbindin-D28K participates in these events remains largely unknown. The potential role of calbindin-D28K in neuronal differentiation has been suggested indirectly, however. For example, expression of calbindin-D28K is increased in PC12 cells following depolarization or treatment with NGF or retinoic acid (5). Several other neurotrophic factors, including neurotrophins and basic fibroblast growth factor, promote the differentiation of certain populations of neurons derived from specific brain regions, and increase or induce the number of cells expressing calbindin (6–10). Furthermore, there appears to be a correlation between the expression pattern of calcium-binding proteins and the differentiation of neuronal shapes (11). However, there have been no direct attempts to investigate whether and how
calbindin-D28K itself involves in neuronal differentiation.

To directly investigate the role of calbindin-D28K in neuronal differentiation, therefore, we first established and characterized a dopaminergic neuronal cell line, MN9D, which overexpressed calbindin-D28K. We further investigated the potential mechanisms by which calbindin-D28K might mediate neuronal differentiation. Here, we report that (i) overexpression of calbindin-D28K itself in MN9D neuronal cells led not only to robust neurite outgrowth, but also to the appearance of a phenotypic marker of maturation, SNAP-25; (ii) neurite outgrowth induced by calbindin-D28K occurred without the cessation of cell division; and (iii) phosphorylation of p38 MAPK was involved in the process of robust neurite extension induced by calbindin-D28K, but not in the expression of SNAP-25; this raises the possibility that calbindin-D28K may play an important role during a specific stage of dopaminergic neuronal differentiation via activation of p38 MAPK.

MATERIALS AND METHODS

Establishment of MN9D cells overexpressing calbindin-D28K. MN9D neuronal cells were previously established by a fusion between murine embryonic mesencephalic neurons and neuroblastoma N18TG (12, 13). For establishing a stable cell line overexpressing calbindin-D28K, MN9D parent cells were plated at 2 × 10⁵ cells onto 25 μg/ml poly-D-lysine-coated six-well plates (Corning, Corning, NY) and transfected with a eukaryotic expression vector, pcDNA3 (Invitrogen; Groningen, The Netherlands; MN9D/Neo) or pcDNA 3 containing a chimeric calbindin-D28K cDNA (a generous gift from Dr. D. R. Dowd at Case Western University; MN9D/Calbindin) using Lipofectamine (Life Technologies, Rockville, MD). Approximately 2 weeks after transfection, G418-resistant colonies were selected and expanded in Dulbecco’s modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum and 250–500 μg/ml G418 (complete culture medium; CCM). Several MN9D/Calbindin clones were characterized with an immunoblot analysis as described below.

Immunoblot analysis. MN9D/Neo or MN9D/Calbindin cells were plated at 1 × 10⁶ cells on poly-D-lysine-coated P-100 dishes (Corning) and maintained in CCM for 3–4 days. If necessary, p38 inhibitor (PD 169316; Calbiochem, San Diego, CA) was included in CCM. Cells were then washed with ice-cold PBS and lysed in a buffer containing 50 mM Tris, pH 7.0, 2 mM EDTA, 1% Triton X-100, 2 mM PMSF, and 10 μg/ml leupeptin and aprotonin for 10 min. Lysates were centrifuged at 13,000g for 30 min at 4°C. Protein contents of the supernatants were measured using the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA). Forty micrograms of proteins from each sample were separated on 12.5% SDS–PAGE, blotted onto pre-wetted PVDF-nitrocellulose filters (Bio-Rad) and further processed for immunoblot experimentation using various antibodies. These included anti-calbindin-D28K antibody which recognized various species of epitope including chicken and mouse (Swant, Bellinzona, Switzerland), mouse monoclonal anti-microtubule associated protein 2 (Roche, Basel, Switzerland), mouse monoclonal anti-NeuN (Swant, Bellinzona, Switzerland), mouse monoclonal anti-5HT-2A receptor (Chemicon International, Temecula, CA), mouse monoclonal anti-calbindin-D28K (Stenberger Monoclonals, Lutherville, MD), and anti-phosphorylated p38, anti-phosphorylated JNK, and anti-phosphorylated ERK antibodies (Cell Signaling, Beverly, MA).

Measurement of neurite outgrowth. MN9D/Neo or MN9D/Calbindin cells were plated at a density of 1 × 10⁵ cells on poly-D-lysine-coated six-well plates (Corning) and maintained in CCM for 1–3 days in the presence or absence of 20 μM PD 169316. The basic pattern of neurite outgrowth was analyzed basically as previously described (14). Briefly, cells from randomly selected areas of two to three culture plates from three independent cultures were photographed. Morphological characteristics were quantitated using an Axiovert Phase-Contrast Microscope equipped with an AxioCam digital camera system and Axiovision image analyzer (Carl Zeiss, Zena, Germany). The pattern of neurite outgrowth was determined on approximately 150–200 MN9D/Neo or MN9D/Calbindin cells. Cell clumps containing more than six cells were excluded from the morphometric analysis. A neurite was defined as a process arising from the soma. The length of the primary neurite was defined as the distance from the soma to the tip of the longest branch; the total extent of neurite was defined as the combined lengths of all neurites per cell. The number of neurites per cell was identified as all processes longer than two cell diameters in length. Cells marked in the same microscopic field were monitored over 4–5 days using an AxioCam digital camera system to determine whether MN9D/Calbindin cells with a robust neurite extension still divide.

RESULTS

To directly assess the potential role of calbindin-D28K in the differentiation of a neuronal cell line, MN9D, cells were stably transfected with a eukaryotic expression vector, pcDNA3 containing a chick calbindin-D28K cDNA (MN9D/Calbindin) and subsequently maintained in complete culture medium for 2 weeks. Among several G-418-resistant colonies, we restricted our studies to two cell lines derived separately, MN9D/Calbindin #27 and #29, which expressed differential levels of chicken calbindin-D28K protein as determined by an immunoblot analysis (Fig. 1A). MN9D cells transfected with a parental vector, pcDNA3 (MN9D/Neo) did not express this protein and were used as a control. As typified in Fig. 1B, only a few, short processes were observed in MN9D/Neo control cells or in the parental MN9D cells (14). In contrast, MN9D/Calbindin stable cell lines exhibited a robust outgrowth of neurites (Fig. 1C). Neuritic outgrowth in MN9D/Calbindin started as early as 24 h after initial plating and extended continuously thereafter. Despite the appearance of a seemingly differentiated morphology in MN9D/Calbindin cell lines, majority of cells with a robust neuritic outgrowth (>70%) still proliferated as demonstrated by time-lapse photographs (Fig. 2). We then quantified and compared these morphological changes with those in MN9D/Neo cells. As shown in Table 1, increases in number of neurites, length of the primary neurite and the total neuritic extent were highly significant in two MN9D/Calbindin cell lines 3–4 days after initial plating. Interestingly, the extent of neurite outgrowth in MN9D/Calbindin #27 was greater than that in MN9D/Calbindin #29, suggesting a close correlation between calbindin-D28K expression and neurite extension. We compared the expression patterns of microtubule associated protein 2 (MAP-2) and synaptosomal-associated protein 25 kDa (SNAP-25) in MN9D/Neo and MN9D/Calbindin cells to investigate whether morphological
differentiation promoted by overexpressed calbindin-D28K in MN9D cells may be accompanied with other hallmarks of neuronal maturation. As demonstrated by immunoblot analysis in Fig. 3, expression level of SNAP-25 increased by at least three-fold in MN9D/Calbindin cells. In contrast, expression level of MAP-2 remained unaltered.

MAPKs are known to be involved in neuronal differentiation in many neuronal cell types. Thus, we examined whether neuritic outgrowth mediated by calbindin-D28K may be associated with activation of specific types of MAPKs in MN9D cells. As shown in Fig. 4, phosphorylation of p38 was slightly increased as early as 1 day after initial plating in MN9D/Calbindin cells and significantly increased 3 days after initial plating when the extent of neurite outgrowth was very apparent. By contrast, the levels and extent of phosphorylation of such other MAPKs as JNK and ERK in MN9D/Calbindin cells remained the same over 1-3 days after initial plating. Consequently, neurite outgrowth in MN9D/Calbindin cells was largely abolished to the level of control MN9D/Neo cells following treatment with a p38 inhibitor, PD 169316 (20 μM; Figs. 5A-5C). Intriguingly, calbindin-D28K-mediated expression of SNAP-25 was not altered by this treatment (Fig. 5D).

**DISCUSSION**

Several molecules and conditions intrinsic to neurons appear to contribute to axonal growth. These include growth-associated proteins, activated cytoskeletal proteins, calcium and its related signals, cAMP, and other second-messenger systems (15-19). In this regard, our present study is novel in two ways. First, we have demonstrated for the first time that overexpression of the calcium-binding protein, calbindin-D28K itself in MN9D dopaminergic neuronal cells leads to robust neuritic outgrowth as typified by increases in number of neurites per cell, primary neurite length and total neurite extent. Interestingly, morphologically-differentiated MN9D cells induced by calbindin-D28K still proliferate. Secondly, the events associated with morphological differentiation seem to be mediated specifically by activation of the p38 MAPK signaling pathway in MN9D cells as phosphorylation of p38 accompanied with these morphological changes. Consequently, treatment with a p38 inhibitor, PD 169316, largely abolishes neurite outgrowth mediated by calbindin-D28K in MN9D cells, implying that the p38 MAPK signaling pathway is one of the major contributors to the neurite outgrowth induced by calbindin-D28K. Previously, we have reported that overexpression of Bcl-2 in MN9D cells induced neurite outgrowth (14). However, phosphorylation of p38 is not detected during Bcl-2 or Bcl-XL-induced neurite out-
growth in MN9D cells (not shown). Therefore, recruitment of the p38 MAPK signaling pathway by calbindin-D28K is a rather specific event in MN9D dopaminergic neuronal cells.

Proteins associated with the presynaptic terminal play critical roles during neurite outgrowth and synaptogenesis. In previous studies by other investigators, it was shown that expression of one of these proteins, SNAP-25, is sufficient to induce neuronal differentiation. For example, both the expression of SNAP-25 and its subcellular distribution are correlated closely with the transformation of growth cones to synaptic terminals (20). Antisense oligonucleotides of SNAP-25 block neuritic elongation in rat cortical neurons and PC-12 cells in vitro, and in amacrine cells of the developing chick retina in vivo (21). Furthermore, overexpression of SNAP-25 itself enhanced neurite elongation in PC12 cells (22). In our present study, treatment of MN9D cells with a p38 kinase inhibitor, PD 169316, reversed calbindin-D28K-mediated neuritic outgrowth to the control levels. However, upregulation of SNAP-25 expression induced by calbindin-D28K was not affected following treatment with PD 169316. Therefore, our present data raise two intriguing possibilities. The first is that upregulation of SNAP-25 itself is not sufficient to induce neurite outgrowth in MN9D dopaminergic neuronal cells. The second is that during neuronal differentiation, there seems to be an additional pathway other than the activation of p38 MAPK by which calbindin-D28K controls such phenomena of biochemical maturations as the expression of SNAP-25.

### TABLE 1

<table>
<thead>
<tr>
<th></th>
<th>MN9D/Neo</th>
<th>MN9D/Calbindin #27</th>
<th>MN9D/Calbindin #29</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of cells</td>
<td>175</td>
<td>175</td>
<td>150</td>
</tr>
<tr>
<td>Number of neurites/cell</td>
<td>1.62 ± 0.13</td>
<td>2.50 ± 0.16**</td>
<td>2.02 ± 0.21*</td>
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<tr>
<td>Primary neurite (µM)</td>
<td>12.33 ± 1.14</td>
<td>49.56 ± 2.35***</td>
<td>23.84 ± 3.11**</td>
</tr>
<tr>
<td>Total neurite extent (µM)</td>
<td>20.85 ± 1.35</td>
<td>73.58 ± 4.79***</td>
<td>41.92 ± 5.37**</td>
</tr>
</tbody>
</table>

Note. MN9D/Neo or MN9D/Calbindin #27 or #29 cells were plated on poly-o-lysine-coated six-well plates at a density of $1 \times 10^5$ and maintained in CCM for 3 days. Three to four randomly selected areas were photographed and morphological characteristics were analyzed from three independent cultures as described under Materials and Methods. All measurements were made without knowledge of cell line. Data were expressed as means ± SEM. Both MN9D/Calbindin cells had significantly more neurites per cell, primary neurite, and total neuritic length than MN9D/Neo cells as determined by Student’s t test: *P < 0.05, **P < 0.005, ***P < 0.0005.

FIG. 3. Immunoblot analysis of SNAP-25 and MAP-2 in MN9D/Neo and MN9D/Calbindin cells. Both MN9D/Neo and MN9D/Calbindin #27 cells plated at a density of $1 \times 10^6$ on poly-o-lysine-coated P-100 dishes, maintained in CCM for 3 days, lysed and processed for immunoblot analysis of SNAP-25 and MAP-2 as described under Materials and Methods. Primary antibodies used were mouse monoclonal anti-SNAP-25 (1:3000) and MAP-2 (1:3000). The blot was from one experiment representative of three similar repeats.

FIG. 4. Immunoblot analysis of the phosphorylated forms of MAPKs in MN9D/Neo and MN9D/Calbindin cells. Both MN9D/Neo and MN9D/Calbindin #27 cells plated at a density of $1 \times 10^6$ on poly-o-lysine-coated P-100 dishes, maintained in CCM for 3 days, lysed and processed for immunoblot analysis of the phosphorylated forms of MAPKs as described under Materials and Methods. Primary antibodies used were rabbit polyclonal anti-phosphorylated forms of p38 (1:1000), JNK (1:1000), and ERK (1:2000). Similar pattern was repeated in at least two to three independent cultures.
Considering the various protein kinases involved in neuronal differentiation, the requirement for MAPKs for neuritic outgrowth is of great interest. It has been well established that activation of the ERK MAPK pathway is crucial for neuronal differentiation. For example, activation of the ERK pathway is necessary and sufficient for NGF- or drug-induced neuronal differentiation of PC12 cells (23–28). It was previously proposed that neuronal survival/differentiation and induction of cell death may be controlled by the opposing actions of the ERK and JNK-p38 MAPK pathways (29). Increasing evidence from several recent studies using PC12 cells for the most part have indicated that activation of the JNK and p38 MAPK signaling pathway not only mediates cellular response to environmental stresses, but is also required for a more widespread set of cellular function including neuritic outgrowth. For example, a specific activation of the p38 MAPK signaling pathway is necessary for neurite outgrowth in response to such growth factors as NGF, EGF and bone morphogenic protein-2 (30, 31). Here, we have demonstrated additionally that signals other than growth factors also activate the p38 MAPK pathway and thereby result in neurite outgrowth in MN9D neuronal cells overexpressing calbindin-D28K. This event is rather specific in that phosphorylation of such other MAPKs as JNK and ERK remains unaltered in MN9D/Calbindin cells. Considering a previous report by other that neurite outgrowth in retinal neurons is not mediated by p38 MAPK pathway (32), therefore, it seems that recruitment of a specific set of MAPKs during neuronal differentiation may depend on cell types and/or the cellular context.

Further studies are needed to elucidate certain critical questions. These questions include (i) whether calbindin-D28K induces neurite outgrowth in a specific population of neuronal cells considering its restricted distribution in neurons in vivo (3); (ii) whether neurite outgrowth in MN9D cells mediated by calbindin-D28K is dependent on its known calcium-buffering function, (iii) how calbindin-D28K-mediated signal(s) converge on the p38 MAPK pathway; and (iv) what target protein(s) downstream of the p38 MAPK pathway act as a final effector of neurite elongation. Answers to these and other critical questions may enlighten our understanding of the potential role for calcium-binding proteins in neuronal differentiation.

**FIG. 5.** Reversal of neurite outgrowth but not of expression of SNAP-25 following treatment with a p38 inhibitor, PD 169316. (A–C) The pattern of neurite outgrowth was measured 3 days after initial plating for both MN9D/Neo and MN9D/Calbindin #27 cells in CCM in the presence or absence of 20 μM PD 169316 as described in Table 1. As determined by Student t test, reversal of (A) number of neurites per cell, (B) primary neurite, and (C) total neurite extent in MN9D/Calbindin #27 cells following treatment with 20 μM PD 169316 was statistically significant; P < 0.0005. (D) Level of SNAP-25 in MN9D/Calbindin #27 cells 3 days after initial plating in the absence of 20 μM PD 169316 (cont) was compared to that in MN9D/Calbindin #27 cells in the presence of 20 μM PD 169316 for various time periods. Immunoblot analysis of SNAP-25 was performed using mouse monoclonal anti-SNAP-25 (1:3000). The blot was a representative of three similar repeats.
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