Borna Disease Virus Persistent Infection Activates Mitogen-activated Protein Kinase and Blocks Neuronal Differentiation of PC12 Cells*

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Persistence of Borna disease virus (BDV) in the central nervous system causes damage to specific neuronal populations. BDV is noncytopathic, and the mechanisms underlying neuronal pathology are not well understood. One hypothesis is that infection affects the response of neurons to factors that are crucial for their proliferation, differentiation, or survival. To test this hypothesis, we analyzed the response of PC12 cells persistently infected with BDV to the neurotrophin nerve growth factor (NGF). PC12 is a neural crest-derived cell line that exhibits features of neuronal differentiation in response to NGF. We report that persistence of BDV led to a progressive change of phenotype of PC12 cells and blocked neurite outgrowth in response to NGF. Infection downregulated the expression of synaptophysin and growth-associated protein-43, two molecules involved in neuronal plasticity, as well as the expression of the chromaffin-specific gene tyrosine hydroxylase. We showed that the block in response to NGF was due in part to the down-regulation of NGF receptors. Moreover, although BDV caused constitutive activation of the ERK1/2 pathway, activated ERKs were not translocated to the nucleus efficiently. These observations may account for the absence of neuronal differentiation of persistently infected PC12 cells treated with NGF.

Borna disease virus (BDV) is a nonsegmented, negative stranded RNA virus (1, 2), belonging to the Bornaviridae family in the Mononegavirales order. BDV causes central nervous system diseases characterized by behavioral abnormalities in a wide variety of animals (3). There is evidence that BDV infects humans, although its role in neuropsychiatric disorders remains a matter of debate (4–9). When inoculated in adult immunocompetent Lewis rats, BDV causes behavioral disturbances associated with massive brain inflammation and extensive neuronal damage (10, 11). Neonatal infection also results in a lifelong persistent infection associated with a variety of behavioral abnormalities but without generalized meningitis or encephalitis (10, 12–15). Therefore, this second model allows studying the consequences of persistent BDV infection on neuronal functions in the absence of inflammation.

Many lines of evidence suggest that BDV persistence per se can cause damage to specific neuronal populations. For example, infection of adults with BDV disrupts cortical cholinergic innervation prior to encephalitis (16), whereas neonatal infection causes alterations in postnatal development of the cerebellum and hippocampus as well as synaptic pathology (13, 14, 17, 18). In particular, neonatal infection causes Purkinje cell death, dentate gyrus granule neuron degeneration, and a progressive loss of cortical neurons (14, 17–19).

BDV is a noncytopathic virus, and the mechanism whereby it causes neuronal pathology is still not understood. Neonatal infection leads to variations in the level of proinflammatory cytokines and to changes in monoamine tissue content as well as in the expression of molecules regulating central nervous system plasticity (18–22). Although these changes might be responsible for part of the pathology, they, however, appear quite late after infection (i.e. at least 3 weeks post-inoculation). A recent study showing that cerebellar damage is observed only if infection occurs before postnatal day 15 suggests that BDV also interferes with early events of postnatal brain development (23). Therefore, we hypothesized that infection may affect the response to neurotrophic factors that play key roles in early differentiation and survival of neurons. In particular, we decided to investigate whether BDV infection interferes with the response to nerve growth factor (NGF), the prototypic member of the neurotrophin family (24). As a first step to test this hypothesis, we studied the response to NGF of PC12 cells infected by BDV. The PC12 cell line is a neural crest-derived adrenal chromaffin cell line obtained from a rat pheochromocytoma. PC12 cells exhibit several features of neuronal differentiation following treatment with NGF. They extend neurites, become post-mitotic, and resemble sympathetic neurons (25). Binding of NGF to its receptor, a tyrosine kinase, triggers a cascade of protein phosphorylation events, leading to the activation of several genes. This signal transduction cascade and the genes involved have been studied extensively (26, 27).

Here, we show that persistent infection of PC12 cells with BDV leads to dramatic changes in cell morphology and expression of genes implicated in synaptic plasticity. Moreover, infection causes a complete block in NGF-induced neurite out-
growth and several changes in the NGF signal transduction cascade.

**EXPERIMENTAL PROCEDURES**

*Maintenance of Cells and Infection with BDV*—PC12 cells (CRL-1721; American Type Culture Collection) were grown at 37 °C in Dulbecco’s modified Eagle’s medium with Glutamax II (Life Technologies, Inc.) supplemented with 10% horse serum, 5% fetal calf serum and 1× Bufferall (Sigma). Cells were passaged once or twice a week using nonenzymatic cell dissociation solution (Sigma). Cells were replated at 1:4 to 1:6 ratio in 75-cm² tissue culture flasks or transferred to culture dishes or glass coverslips coated with collagen (Sigma). PC12 cells were infected with BDV CRP4 as described (13) and subcultured 3 days after the initial infection to establish a persistently infected line (PC12-BV). PC12-BV cells were subcultured (1:5) every 4–6 days under the same conditions as the noninfected control cells.

*Treatment with NGF*—Infected and noninfected PC12 cells were plated on collagen-coated supports, grown in complete medium for 16 h, and starved in medium containing 1% serum for 16 h. They were then treated with 100 ng/ml NGF (Life Technologies, Inc.), for the indicated times before analysis.

**Northern Blot Analysis**—Total RNA was isolated using TRI-Reagent (Sigma), size-fractionated by 2.2 M formaldehyde-agarose gel electrophoresis, transferred by capillarity with 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) to Magnagraph nylon membranes (MSI, Westboro, MA), and UV cross-linked. The blots were hybridized for 2.5 h at 65 °C in Quikhyb buffer (Stratagene, La Jolla, CA) containing 100 μg/ml and 5 ng/ml of heat-denatured salmon sperm DNA and RNA probe, respectively. RNA probes were labeled with [α-32P]dCTP using random hexamers (Prime-it II; Stratagene, La Jolla, CA). After high stringency washes (0.2× SSC, 0.2% SDS at 65 °C, twice), the blots were exposed to Biomax MS films (Eastman Kodak Co.). After each hybridization, the blots were stripped by boiling twice in stripping buffer (2 mM EDTA, 5 mM Tris, pH 7.5, 0.1% SDS) before rehybridized. The following probes were used: cDNA fragments from the BDV nucleoprotein (NP), rat synaptophysin, GAP-43, and tyrosine hydroxylase (28) genes, as well as cDNA fragments from rat p75 and TrkA receptors (gifts from C. Tuffereau and M. Barbacid, respectively). The following probes were used: cDNA fragments from the ERK-1/ERK2 (diluted 1:500), MAPK-1 (diluted 1:200), microtubule-associated protein (MAP)-2 (diluted 1:2500) described previously (6).

**Immunofluorescence and Confocal Microscopy Analysis**—Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline followed by methanol-acetone (50/50) and processed for immunofluorescence as described previously (30). Briefly, after blocking with 2% normal goat serum and 2% horse serum for 1 h at room temperature, cells were stained with primary antibodies. After washing in phosphate-buffered saline containing 0.1% Triton X-100, cells were stained with secondary antibodies, i.e. fluorescein isothiocyanatelabeled anti-rabbit IgG (Interchim; diluted 1:500) and Cy3-labeled anti-mouse IgG (Interchim; diluted 1:500). Cells were extensively washed as described for primary antibodies, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and sealed with nail polish. The primary antibodies were monoclonal antibodies to synaptophysin (Roche Molecular Biochemicals; diluted 1:5), microtubule-associated protein (MAP)-2 (Sigma; diluted 1:200), βIII-tubulin (Sigma; diluted 1:200) and NGF receptor molecule p75 (Roche Molecular Biochemicals; diluted 1:10). We also used a rabbit polyclonal antibody to the Tau protein (Sigma; diluted 1:200), as well as the antibodies to the phosphorylated forms of ERK-1/ERK2 (diluted 1:200) and to BDV NP (diluted 1:25000) described above.

**Immunofluorescence and Confocal Microscopy Analysis**—Cells grown on glass coverslips were fixed with 4% paraformaldehyde in phosphate-buffered saline followed by methanol-acetone (50/50) and processed for immunofluorescence as described previously (30). Briefly, after blocking with 2% normal goat serum and 2% horse serum for 1 h at room temperature, cells were stained with primary antibodies. After washing in phosphate-buffered saline containing 0.1% Triton X-100, cells were stained with secondary antibodies, i.e. fluorescein isothiocyanate-labeled anti-rabbit IgG (Interchim; diluted 1:500) and Cy3-labeled anti-mouse IgG (Interchim; diluted 1:500). Cells were extensively washed as described for primary antibodies, mounted in Vectashield (Vector Laboratories, Burlingame, CA), and sealed with nail polish. The primary antibodies were monoclonal antibodies to synaptophysin (Roche Molecular Biochemicals; diluted 1:5), microtubule-associated protein (MAP)-2 (Sigma; diluted 1:200), βIII-tubulin (Sigma; diluted 1:200) and NGF receptor molecule p75 (Roche Molecular Biochemicals; diluted 1:10). We also used a rabbit polyclonal antibody to the Tau protein (Sigma; diluted 1:200), as well as the antibodies to the phosphorylated forms of ERK-1/ERK2 (diluted 1:200) and to BDV NP (diluted 1:3000) described above. When performing double staining, primary and secondary antibodies were used simultaneously. After staining, cells were examined with a TCS4D Leica laser scanning confocal microscope. Digitalized images were processed using Adobe Photoshop and Canvas softwares.

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RESULTS

Persistence of BDV in PC12 Cells Causes Progressive Changes in Cell Morphology—PC12 cells were infected with BDV as described above and analyzed at each passage after infection (Fig. 1). Early after infection, infected cells were morphologically undistinguishable from control noninfected PC12 cells. After three passages, over 96% of the cells were infected, as determined by detection of the viral nucleoprotein using immunofluorescence and fluorescence-activated cell sorter analysis (not shown). After four to five passages, cells in infected cultures changed morphology. PC12-BV cells became flat and nonrefractile, and their adhesion to the support increased (Figs. 1 and 2). After eight to 12 passages (depending on the experiment), the changes concerned the whole cell population.

We also examined the response of infected PC12 cells to NGF. NGF blocks the proliferation of noninfected PC12 cells and induces neurite outgrowth. As shown in Fig. 2, PC12-BV cells treated with NGF failed to develop neurites and continued to proliferate. By contrast, control PC12 cells passaged in parallel remained unchanged and responded normally to NGF (Fig. 2). Five independent experiments gave similar results. The infection did not impair cell viability, as determined by trypan blue staining (not shown). Infected cells did not lose BDV upon passaging, and viral infection did not impair cell growth. The doubling time of PC12-BV cells under normal serum conditions was slightly shorter than that of control cells (44.6 ± 2 h. versus 65 ± 3 h., n = 9, p < 0.0005 by Mann-Whitney test).

We next asked whether NGF-treated PC12-BV cells still expressed neuronal markers. Cells were grown on coverslips, treated with NGF, fixed, and processed for immunofluorescence. Compared with control PC12 cells, PC12-BV cells expressed comparable levels of MAP-2, Tau protein, and neuron-specific βIII-tubulin. The expression of MAP-2 appeared even higher in PC12-BV cells than in the control cells (Fig. 3). In contrast, the expression of synaptophysin, a presynaptic vesicle marker, was lost in PC12-BV cells (Fig. 4). Interestingly, synaptophysin expression was lost progressively as PC12-BV cells were passaged. Loss of synaptophysin expression correlated with the morphological alterations linked to BDV persistence (Fig. 4). At later passages, very few cells still expressed synaptophysin and responded to NGF. These cells were, in fact, not infected (Fig. 4, bottom panels). All experiments described in the rest of this study were performed on persistently infected cells that had undergone all of the above-described phenotypic changes.

Impaired Gene Expression in PC12 Cells Infected with BDV—We then examined the level of mRNA coding for synaptophysin in PC12 and PC12-BV cells, together with the level of mRNA encoding another plasticity-related protein, the GAP-43 (31). We also examined the expression of the tyrosine hydroxylase (TH) gene, a delayed-early gene induced by NGF in PC12 cells (32). TH is an enzyme that catalyzes the rate-limiting step in the catecholamine biosynthetic pathway leading to dopamine synthesis. It is expressed in catecholaminergic neurons as well as in neural crest-derived chromaffin cells. Finally, Northern blots were also probed for the housekeeping gene GAPDH and for viral RNA.

FIG. 2. NGF does not induce neurite outgrowth in PC12 cells persistently infected with BDV (PC12-BV). PC12 or PC12-BV cells (passage 10 after infection) were grown on collagen-coated plates for 3 days in the presence or absence of 100 ng/ml NGF. The cells were fixed and briefly stained with Giemsa.

FIG. 3. Expression of neuronal-specific markers in PC12 and PC12-BV cells. Cells were grown on collagen-coated coverslips for 2 days in the presence of NGF, fixed, and processed for immunofluorescence for the detection of MAP-2, Tau, and βIII-tubulin.

FIG. 4. BDV infection causes a progressive down-regulation of synaptophysin expression. Control PC12 cells and cells infected with BDV and passaged 1, 6, and 9 times (P1, P6, and P9) are shown. PC12 and PC12-BV P9 cells were treated with NGF for 4 days. PC12-BV P1 and P6 were left untreated. All cells were doubly stained for synaptophysin (red) and BDV NP (green). The higher density of PC12-BV (P9) cells is due to the fact that these cells do not become post-mitotic in response to NGF. Infected cells stained with the BDV NP antibody exhibit a characteristic punctuate staining of the nucleus (better seen on Z series scans; not shown), together with a diffuse cytoplasmic staining. Note the progressive decrease of synaptophysin in infected cells. At passage 9, synaptophysin is not detected in PC12-BV cells, except for a single cell that is not infected (see merged images).
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Fig. 5. Impaired mRNA expression in PC12 cells persistently infected with BDV (PC12-BV). RNA was extracted from PC12 and PC12-BV cells treated or not with NGF for the indicated times and analyzed by Northern blot hybridization with probes for synaptophysin (Syn), GAP-43, TH, BDV NP, and GAPDH. Exposure times for autoradiography vary depending on the probe and range from 4 to 30 h. Staining with ethidium bromide (EtBr) shows the integrity and the amount of ribosomal 28 and 18S loaded in each lane. Hybridization with a probe for the housekeeping GAPDH gene provided another control for the integrity of mRNA. Similar results were obtained in three independent experiments.

As shown in Fig. 5, PC12 cells expressed relatively high constitutive levels of synaptophysin, and exposure to NGF was accompanied by the characteristic increase (33) in GAP-43 RNA levels. Likewise, TH mRNA was readily detected in untreated PC12 cells, and expression increased following NGF treatment. In contrast, mRNAs for synaptophysin, TH and GAP-43 were not, or barely, observed by Northern blotting in PC12-BV cells and were not up-regulated in response to NGF (Fig. 5), whereas mRNA levels for GAPDH remained unaffected. As expected, we only detected BDV-specific RNAs in PC12-BV cells. Hence, BDV persistence in PC12 cells specifically alters the expression of genes coding for molecules involved in synaptic plasticity as well as in catecholamine biosynthesis.

Expression of NGF Receptors Is Down-regulated in BDV-infected PC12 Cells—We next investigated whether the absence of response of PC12-BV cells to NGF could be due to a down-regulation of the expression of the NGF receptors. Two structurally unrelated NGF receptors are known (34). The low affinity neurotrophin receptor p75 is a transmembrane glycoprotein that also binds the other neurotrophins BDNF, NT3 and NT4/5. NGF also binds and activates the tyrosine kinase receptor TrkA, and it has been suggested that both p75 and TrkA may be required for the formation of the high affinity NGF-binding site and for the full NGF-mediated effect (34, 35).

We analyzed the expression of mRNAs encoding p75 and TrkA using Northern blot (Fig. 6A). In PC12 cells, we observed a significant increase in the level of mRNAs encoding p75 and TrkA molecules following NGF treatment. In contrast, in PC12-BV cells, mRNAs levels were greatly decreased for p75 and were below the threshold of detection for TrkA. We next studied the expression of p75 by indirect immunofluorescence (Fig. 6B). The p75 molecule was strongly expressed after NGF treatment in PC12 cells, whereas expression was uneven in BDV-infected PC12 cells. In the latter, some cells had levels of p75 comparable with those of the noninfected cells, whereas others had levels that were barely detectable. Levels of p75 did not appear to correlate with viral nucleoprotein load. TrkA expression was below the threshold of detection by immunofluorescence, even in the control PC12 cells.

Alterations in NGF Signal Transduction Cascade in PC12 Cells Infected with BDV—The block in neuronal differentiation observed in PC12-BV can be explained in part by the down-regulation of NGF receptor expression. However, results from Northern blot and immunofluorescence showed that some PC12-BV cells still expressed low levels of receptor (at least for p75) and nevertheless did not differentiate in response to NGF.

Moreover, analysis of mRNA expression with a sensitive reverse transcriptase-polymerase chain reaction assay showed that infected cells still expressed low levels of TrkA mRNA (data not shown). Therefore, we decided to examine the effects of BDV infection on the NGF signal transduction cascade (27) (Fig. 7A). Binding of NGF to the TrkA receptor triggers sequential phosphorylations that propagate the signal to the nucleus. Phosphorylation of the MAP/ERK kinases (MEK) isoforms (the 45-kDa MEK1 and the 46-kDa MEK2) stimulates the mitogen activated protein kinases ERK1 and ERK2 (p42 and p44 MAPK). Following their activation, the ERKs phosphorylate a large number of regulatory proteins including the transcription factor Elk-1 that will subsequently bind to serum response elements located in several promoters (36). In addition, the ERKs will phosphorylate the p90RSK kinase (37), that will in turn phosphorylate targets such as CREB and activate the transcription of immediate early genes such as c-fos by binding to cAMP response element-binding sites (38, 39).

The existence of commercially available antibodies specific for activated MAPK proteins (antibodies that will only recognize double-phosphorylated substrates) allowed us to study in detail the effects of BDV infection on the NGF signal transduction cascade. Because most events occur within minutes after addition of NGF, PC12 and PC12-BV cells were rapidly lysed at different times after adding NGF, and equal amounts of proteins (as determined by Bradford assays) were analyzed by...
Western blot for the expression of the above-mentioned activated kinases. In some cases, the blots were stripped after detection of the activated form of the kinase and reprobed with an antibody that detects all forms of the kinase (phosphorylated and nonphosphorylated). This experiment was repeated at least five times, and the results were consistently similar to those shown in Fig. 7B. We observed no basal expression of activated MEK or ERK in control PC12 cells prior to adding NGF and only minimal CREB phosphorylation at this point. After adding NGF, both MEK and ERK were quickly and strongly induced. This was followed by activation of Elk1 and of the p90RSK/CREB cascade and ultimately by transcription of c-fos, the latter being analyzed by Northern blotting (Fig. 7B). Several marked differences were observed in PC12 cells persistently infected with BDV (Fig. 7B). First, we observed that MEK1/2 and ERK1/2 were constitutively activated, even in the absence of NGF. This was consistently observed, even if cells were serum-starved for long periods of time (not shown). It should be emphasized that activated forms of MEK and ERK were never observed in noninfected PC12 cells before NGF treatment, even after increasing the quantity of protein on the blot. Second, PC12-BV cells responded to NGF treatment with an increase in the amount of activated MEK and ERK proteins to levels comparable with those of the control PC12 cells. This strongly suggests that the TrkA receptor molecule is still present in PC12-BV cells and is capable of initiating the MAPK signaling cascade. Nevertheless, this increased activation was more transient than in control PC12 cells and levels returned rapidly to baseline. Third, activation of the p90RSK/CREB pathway was significantly decreased, leading to only low level of c-fos transcription. Fourth, there was constitutive phosphorylation of Elk1 in PC12-BV cells, and treatment with NGF caused accelerated additional phosphorylation of this transcription factor.

The Nuclear Translocation of Phosphorylated ERKs Is Impaired in BDV-infected PC12 Cells—The finding that the MAPK pathway is constitutively activated in PC12-BV cells is in apparent contradiction with the fact that these cells do not differentiate (40). However, we also observed that activation of the p90RSK/CREB cascade was decreased in PC12-BV cells. Because this activation requires the nuclear translocation of activated ERKs, we hypothesized that BDV infection may interfere with the nuclear translocation of activated ERK1/2. To examine this possibility, PC12 and PC12-BV cells were plated on collagen-coated coverglasses, treated with NGF, and analyzed for the localization of the activated ERK1/2 proteins by indirect immunofluorescence and confocal microscopy (Fig. 8A). The results shown are representative of a large number of cells analyzed for each time point, and the experiment was repeated several times. Consistent with the results of Western blots, there was no detectable levels of activated ERK1/2 in PC12 cells prior to NGF treatment. As early as 5 min after NGF addition, activated ERK1/2 was strongly expressed and was localized both in the nucleus and in the cytoplasm of PC12 cells. The vast majority of cells were strongly positive for activated ERKs by 10 min after treatment. In most of them, the signal was mainly nuclear. Rapidly thereafter, the signal decreased, and by 4 h the signal was barely detectable. In PC12-BV cells, activated ERKs expression was again detected even prior to NGF treatment, confirming our Western blot results. Expression levels were variable from cell to cell, and the proportion of cells strongly expressing ERKs increased upon NGF treatment. By 30 min, levels were back to those observed before NGF. Interestingly, the distribution of activated ERKs staining was very different from that in control cells. Confocal analysis (by image overlay and three-dimensional scan, also performed after staining with a nuclear-specific dye; data not shown) revealed that staining was almost exclusively cytoplasmic and

FIG. 7. Analysis of the MAPK signaling pathway in PC12 and PC12-BV cells. A, schematic representation of intracellular events triggered by NGF binding (see text for details). B, Western blot analysis of phosphorylated (P) or nonphosphorylated forms of the different kinases and Northern blot analysis of c-fos transcription. Total protein extracts were rapidly processed at different times after NGF treatment (indicated above the blots), and equal amounts of protein (10 μg for the detection of ERK and NP, 20 μg for detection of MEK, and 30 μg for the detection of CREB, ribosomal S6 kinase, and Elk-1) were analyzed in parallel for PC12 and PC12-BV cells as described under “Experimental Procedures.” For the detection of ERK and CREB, expression of activated (phosphorylated) proteins was analyzed first, and then total ERK or CREB protein were immunodetected on the same blot after stripping. BDV infection was assessed by immunodetection of the viral NP. Arrows on the right of each blot indicate the expected position of the different proteins. Each blot is representative of at least five independent experiments. Moreover, mRNA was analyzed by Northern blotting for c-fos expression in three independent experiments, with one representative result being shown in the figure (bottom panel).
that there was very little activated ERK translocated to the nucleus of PC12-BV cells.

We examined whether these changes were already present in short term infected PC12 cells. As shown in Fig. 8B, the nuclear relocational of activated ERK was already impaired in the rare infected cells present in the population early after infection. Moreover, some infected cells were already resistant to NGF-induced neurite outgrowth. Therefore, the changes occur early after infection and spread in time, together with the virus, to the entire cell population.

DISCUSSION

It has been proposed that the neurological symptoms observed in rats infected at birth with BDV are linked to alterations of the morphogenesis of the hippocampus and cerebellum, two brain structures that continue to develop after birth (19, 41). It is well established that neurotrophins such as NGF have a profound influence on the development of the central nervous system (24). The critical role of neurotrophins in supporting neuronal differentiation and protecting from neuronal programmed cell death, together with recent results suggesting that BDV neonatal persistent infection causes alterations of synaptic plasticity and neuronal cell death by apoptosis (18, 19, 22), led us to examine the effects of BDV on NGF signaling. Because BDV is noncytopathic, it persists and replicates at high levels in neurons in vivo, as well as in PC12 cells in vitro. Therefore, this cell line is an interesting model to study the effects of viral infection on neuronal gene expression and on the responses to neurotrophic factors. Here, we describe an experimental system suitable to explore the mechanisms whereby persistent BDV infection leads to decreased neuronal gene expression and differentiation.

We observed that the persistence of BDV in PC12 cells was accompanied by a progressive change of phenotype. Although PC12 mutant cells with altered responses to NGF can arise spontaneously during continuous culturing (42, 43), we can rule out that the phenotype described here was due to culturing conditions and clonal selection of PC12 cells because (i) we never observed a similar change of phenotype in control PC12 cells maintained under the same conditions and for the same number of passages and (ii) a similar change of phenotype was obtained in several independent experiments. Moreover, none of the PC12 mutants reported so far exhibit the pattern of gene expression and receptor tyrosine kinase activity observed in PC12-BV cells. Finally, at early stages infected cells were morphologically undistinguishable from control cells and expressed normal levels of synaptophysin. This argues strongly against initial infection of a cell subpopulation with characteristics different from those of normal PC12 cells. Instead, it is most likely that viral persistence in PC12 caused a progressive change of phenotype.

BDV infection did not change the level of expression of neuronal markers normally expressed in PC12 cells, such as Tau or βIII-tubulin. The increased expression of MAP-2 in PC12-BV cells may be due to cytoskeleton rearrangements accompanying the change of morphology and adhesion properties caused by BDV. Alternatively, it may be due to the increased activation of ERKs, because activated ERKs can interact with MAP-2 (44). In contrast, the expression of neuronal plasticity-related proteins such as synaptophysin and GAP-43 was severely impaired in PC12-BV. This is consistent with our previously reported results showing impaired synaptophysin and GAP-43 protein expression in the central nervous system of neonatally infected rats (18). The expression of TH was also inhibited in PC12-BV cells. Taken together, these results suggest that BDV infection down-regulates a set of neuronal-specific genes linked...
to NGF signaling, without affecting NGF independent neuronal gene expression.

BDV-infected PC12 cells failed to extend neurites in response to NGF. We showed that the block of NGF signaling was due in part to a strong down-regulation of NGF receptor expression. We then examined the effects of BDV infection on receptor tyrosine kinase signaling and immediate early gene induction, expecting that the MAPK signaling pathway would also be decreased. In contrast, we identified several important changes, which can be summarized as follows: MEK1/2 and ERK1/2 proteins were constitutively activated and responded transiently to NGF induction; Elk-1 transcription factor activation followed the same pattern; and phosphorylation of the components of the p90RSK/CREB cascade was decreased and led to little or no c-fos mRNA induction.

The MAPK signaling cascade is implicated in the response of cells to several growth factors and mitogens. In the case of PC12 cells, the best-studied models have been the response to NGF and to epidermal growth factor (EGF). Although NGF causes neurite extension and acquisition of a post-mitotic phenotype, EGF treatment leads instead to proliferation mediated by the EGF receptor, which is also a tyrosine kinase. Both TrkA and EGF receptor induce similar early transduction pathways, with a strong activation of ERK proteins. The key bifurcation between EGF and NGF-mediated signaling pathways lies in the duration of ERK2 activation that they induce. Transient activation of the ERK pathway will induce proliferation, whereas sustained ERK activation is critical for differentiation of PC12 cells (40). Hence, we were surprised to find that BDV infection caused a sustained activation of ERKs, which was not followed by activation of immediate early genes and neuronal differentiation. A detailed analysis of signaling events that follow the phosphorylation of ERKs, including the phosphorylation of Elk-1, p90RSK, and CREB, indicated that the ERKs followed the phosphorylation of ERKs, including the phosphorylation of Elk-1, p90RSK, and CREB, activated by BDV infection. A detailed analysis of signaling events that follow the phosphorylation of ERKs, including the phosphorylation of Elk-1, p90RSK, and CREB, indicated that the ERKs failed to transduce a strong nuclear signal in BDV-infected PC12 cells. In particular, the expression of phosphorylated CREB, which localizes exclusively to the nucleus, was decreased in PC12-BV cells, suggesting that ERK1/2 might not efficiently translocate to the nucleus. The low level of CREB activation remaining in infected cells could be due to phosphorylation by MSK-1 (45). The impaired translocation of ERKs to the nucleus of infected cells was confirmed by immunodetection of activated ERKs and analysis by confocal microscopy. For Elk-1, however, we observed a constitutive activation (similar to the MEKs and ERKs proteins) as well as an accelerated response after treatment with NGF. This may be due to the fact that this transcription factor, which is expressed mainly in neuronal cells, localizes both to the cytoplasm and to the nucleus before activation (46). It may also be due to increased MAPK-dependent phosphorylation of Elk1 (47).

The precise mechanism leading to chronic activation of ERK1/2 in PC12-BV cells is not known. We were unable to detect ERK1/2 activation following treatment of PC12 cells with supernatants from PC12-BV cells (data not shown). This suggests that cytokine and/or soluble factors released by the infected cells are not responsible for an autocrine loop leading to chronic ERK1/2 activation. A possible explanation is that BDV replication affects the regulation of the phosphorylation or dephosphorylation processes in the cell. For example, BDV infection could enhance the activity of any of the components of the Ras-dependent pathway. Similar to all the nonsegmented, strandless-arranged RNA viruses, BDV encodes a phosphoprotein that is phosphorylated by the host cellular machinery. Although phosphorylation of the BDV phosphoprotein does not appear to be related to the ERK1/2 pathway (48), it could nevertheless interfere with up-stream events involved in phosphorylating ERK1/2. Alternatively, phosphorylation of ERK1/2 is normally mediated by a substrate-specific MAPK phosphatase (MKP-1), which is present in the nucleus (49). This phosphatase rapidly dephosphorylates the activated ERKs after their nuclear translocation, allowing their recycling to the cytoplasm. It is possible that impaired nuclear translocation leads to a progressive cytoplasmic accumulation of activated ERK in PC12-BV cells. How BDV alters nuclear translocation of ERK remains to be established. Because it actively replicates in the nucleus, transport of viral RNA and proteins is an important feature of the BDV life cycle. It implicates viral proteins such as the nucleoprotein, the phosphoprotein and the protein X/p10, whose interactions have been well studied (50–52). However, cellular factors associated with these viral proteins in the nuclear import of viral RNP have not been identified yet. One can hypothesize that BDV could hijack some cellular factors linked to nuclear import and divert them from their normal functions, hence affecting the nuclear translocation of proteins such as the activated ERKs. To our knowledge, this is the first description of a persistent viral infection interfering with neuronal differentiation and linked to impaired ERK nuclear translocation. It has been demonstrated that adenovirus type 5 E1a protein causes a block of NGF differentiation, in this case linked to association of E1a with proteins implicated in cell cycle regulation (53, 54). Interestingly, it has recently been shown that the ret oncopene also blocks the nuclear translocation of ERK and renders PC12 cells resistant to NGF (55).

In the central nervous system, the Ras/ERK pathway and MAPK-dependent transcription are important regulators of neuronal survival and synaptic plasticity (56). If such pathways are indeed affected in BDV-infected animals, this may lead to severe dysfunction, particularly in the critical phases of early postnatal development.

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