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Short Communication

Defective NF-κB activation in virus-infected neuronal cells is restored by genetic complementation

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The interferon-beta (IFNβ) gene is not inducible in neuronal cells in response to measles virus (MV) due to lack of nuclear factor kappa B (NF-κB) activation. NF-κB is normally sequestered in the cytoplasm by an inhibitor ($I\kappa B\alpha$). Previously, the authors demonstrated that the failure to activate neuronal NF-κB by MV was due to the inability to phosphorylate and degrade its inhibitor, $I\kappa B\alpha$. Here the authors demonstrate that transient transfection of a brain cDNA library into neuronal cells restores the ability of MV to activate NF-κB. In addition, tumor necrosis factor-alpha (TNFα), but not interleukin-1 (IL-1) or lipopolysaccharide (LPS), stimulation resulted in $I\kappa B\alpha$ phosphorylation and degradation in two neuronal cell lines. These results indicate that failure of MV to activate neuronal NF-κB is due to a signaling defect and that MV utilizes an NF-κB signaling pathway distinct from that of TNFα, but may overlap with that for IL-1 and LPS. *Journal of NeuroVirology* (2002) **8,** 459–463.

Keywords: interferon-beta; $I\kappa B\alpha$; measles virus; neuronal cell; NF-κB

Viral infection of the central nervous system (CNS) produces a variety of disorders, including acute and chronic persistent infections. Neurons, in particular, appear to foster viral persistence, but the mechanisms responsible for persistence are not completely understood. Because human leukocyte antigen (HLA) class I expression is normally limited in the CNS, one mechanism for virus persistence implicates the lack of HLA class I expression in virus-infected neurons, thus allowing escape from recognition by HLA class I–restricted cytotoxic T cells (CTLs) (Joly *et al*, 1991; Dhib-Jalbut and Johnson, 1994). Measles virus (MV) is a human neurotropic virus that can produce persistence in neurons in patients with subacute sclerosing panencephalitis (SSPE) (Dhib-Jalbut and Johnson, 1994) or subacute measles encephalitis (Liebert *et al*, 1988). We had previously demonstrated that MV infection of glial and endothelial cells upregulates HLA class I expression, primarily through the production of interferon-beta (IFNβ) (Dhib-Jalbut and Cowan, 1993). In contrast, MV was unable to induce HLA class I molecules and IFNβ in neuronal cell lines (Dhib-Jalbut *et al*, 1995). This finding was not due to the transformed nature of the neuronal cell lines, because MV infection of neurons in human SSPE and in an animal model of MV encephalitis also failed to induce major histocompatibility complex (MHC) molecules on the infected cells and IFNβ was not detected in the infected neurons (Gogate *et al*, 1996). The failure to induce HLA class I on neuronal cells was associated with a lack of induction of IFNβ and a failure to activate nuclear factor kappa B (NF-κB) DNA-binding activity, an event critical for induction of the IFNβ gene by virus (Dhib-Jalbut *et al*, 1995). In addition, because IFNβ is an antiviral cytokine, lack of its production in virus-infected neurons provides a novel escape route that could favor viral persistence in neurons. More recently, we have demonstrated that the inability of MV to activate neuronal NF-κB is due to a lack of phosphorylation and degradation of the NF-κB inhibitor, IκBα (Dhib-Jalbut *et al*, 1999).

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NF-κB is a transcription factor necessary for virus-induced IFNβ gene transcription in a variety of cell types (Hiscott *et al*, 2001). NF-κB consists of heterodimeric proteins (p50 and p65) and is normally sequestered in the cytoplasm by an inhibitor (IκBα). Stimulation with a variety of agents results in the phosphorylation and degradation of IκBα, thus freeing up NF-κB, which is then translocated to the nucleus, binds to a target promoter, and induces transcription (Finco and Baldwin, 1995). We have recently demonstrated that lack of NF-κB induction by MV in neuronal cells was not due to excess synthesis of the inhibitor IκBα or an abnormal MV receptor (Fang *et al*, 2001). Therefore, we examined the hypothesis that failure to activate neuronal IκBα is due to a signaling defect, probably involving factor(s) that normally would induce IκBα phosphorylation and degradation.

$I_\kappa B_\alpha$ sequence analysis

In order to determine whether failure of MV to phosphorylate neuronal IκBα is due to a mutation, we sequenced reverse transcriptase–polymerase chain reaction (RT-PCR)–amplified IκBα cDNA from the neuronal and glial cells and compared the sequence to that of wild-type IκBα as described by Haskil and Baldwin (1991). The sequence was not different among U251, IMR32, or the published wild-type IκBα coding sequence (data not shown).

Neuronal and glial $I_\kappa B_\alpha$ mRNA steady-state levels

In order to rule out the possibility that lack of neuronal NF-κB activation is due to increased steady-state IκBα levels or enhanced IκBα synthesis in response to virus stimulation, we compared IκBα mRNA levels in neuronal and glial cells as described earlier (Dhib-Jalbut and Cowan, 1993). IκBα levels were normalized against mRNA levels for the housekeeping gene G3DPH. A representative Northern blot and mean IκBα/G3DPH mRNA from two experiments are shown in Figure 1. First, we observed that steady-state IκBα levels in neuronal cells did not exceed those in glial cells. Second, IκBα enhancement in response to TNFα was observed in both cell types, which is consistent with the ability of TNFα to induce NF-κB in both neuronal and glial cells and with the fact that NF-κB activation induces IκBα synthesis (Finco and Baldwin, 1995). However, IκBα mRNA levels were enhanced in response to MV stimulation in glial but not in neuronal cells, consistent with the lack of NF-κB activation in these cells. Therefore, failure of MV to activate neuronal NF-κB was not due to increased IκBα steady-state levels or enhanced synthesis in response to MV stimulation.

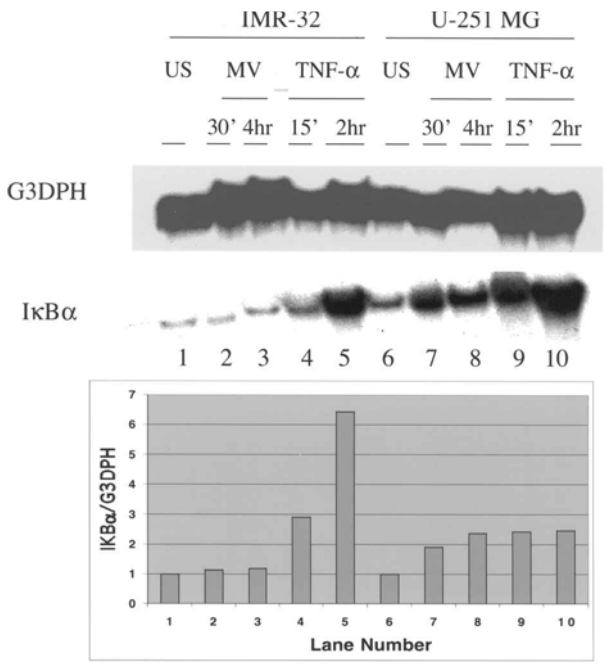

Figure 1 Northern blot of IκBα mRNA in neuronal (IMR-32) and glial (U251 MG) cells in response to MV or TNFα stimulation as described by Dhib-Jalbut and Cowan (1993). The IκBα probe was generated by PCR as described (Haskil and Baldwin, 1991). Treatment conditions included unstimulated (US) cells, cells harvested 30 min after MV stimulation or 4 h later, and TNFα stimulation for 15 min or 2 h later. The intensities of the bands were quantitated by densitometry, normalized for lane-to-lane variation against the control G3DPH mRNA, and expressed relative to the unstimulated condition for each cell line. The bar graph represents mean values from two experiments.

Differential $I_\kappa B_\alpha$ degradation in neuronal and glial cells in response to IL-1 and LPS

Interleukin-1 (IL-1), lipopolysaccharide (LPS), and tumor necrosis factor-alpha (TNFα) are potent activators of NF-κB. Although all utilize the IκB-inducing kinase (IKK) complex to stimulate IκBα phosphorylation, evidence indicates that kinases upstream of IKK are different (Nasuhara *et al*, 1999). Because the neuronal cells activate NF-κB in response to TNFα, but not MV, stimulation, we examined the response to IL-1 and LPS in order to gain an insight into the factors critical for IκBα phosphorylation that might be deficient in neuronal cells. Cells were exposed to the different stimuli for 15 min (an optimal time point that captures IκBα phosphorylation as determined by the presence of a slower migrating band). As shown in Figure 2, MV, TNFα, IL-1, and LPS all resulted in IκBα phosphorylation and degradation in the glial cells. The double bands observed in response to MV, TNFα, and IL-1 indicate phosphorylation of IκBα, which precedes its degradation (Dhib-Jalbut *et al*, 1999). In contrast, in neuronal cells, only TNFα stimulation resulted in IκBα degradation. Therefore, as with MV, IL-1 and LPS failed to induce IκBα degradation in

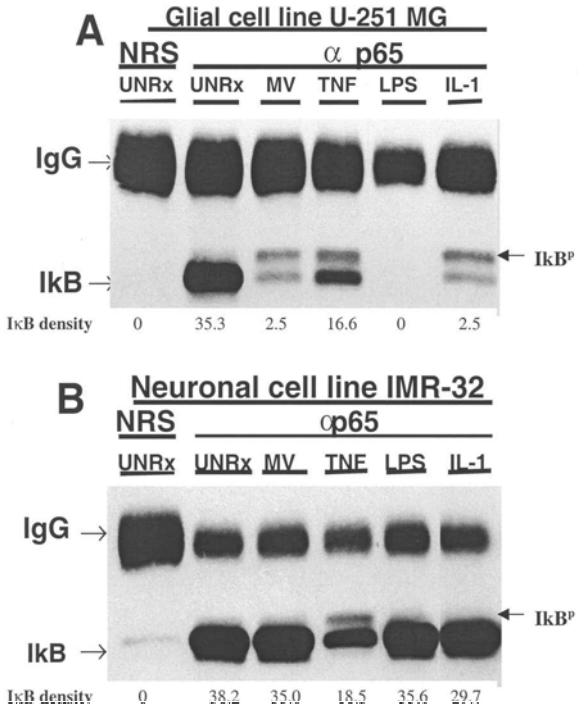

Figure 2 Differential IκBα degradation in response to MV, IL-1, and LPS in glial and neuronal cells. The figure represents a 15% SDS-PAGE followed by Western blot analysis for IκBα degradation in response to MV, TNFα, IL-1, and LPS in the neuronal (IMR-32) and glial (U251 MG) cells. IκBα was coimmunoprecipitated with anti-p65 NF-κB subunit and the blots were probed with anti-IκBα (Santa Cruz, CA) (Dhib-Jalbut *et al*, 1999). In contrast to TNFα stimulation, MV, IL-1, and LPS induced IκBα phosphorylation and degradation in glial, but not neuronal, cells. The slower migrating band observed in response to MV, TNFα, and IL-1 in glial cells reflect phosphorylated IκBα. NRS: normal rabbit serum; UNRx: untreated; αp65: antibody to the p65 subunit of NF-κB.

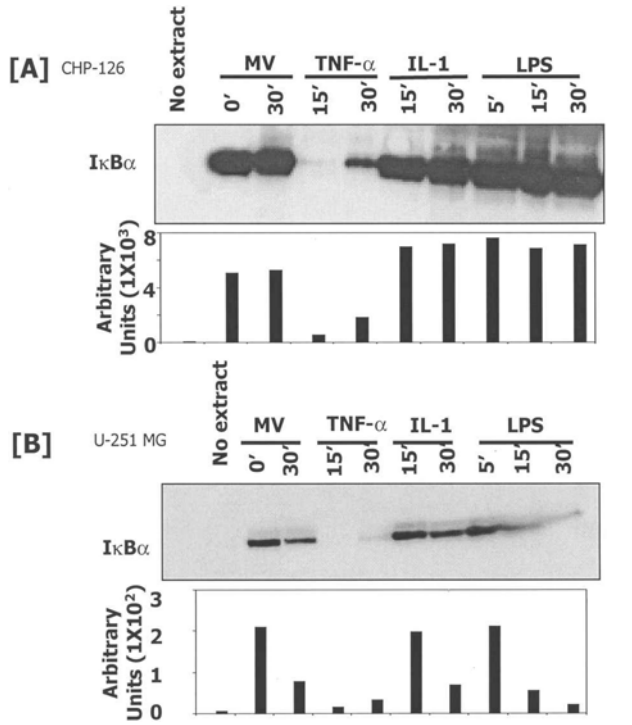

Figure 3 Western blot analysis of IκBα degradation in response to MV, TNFα, IL-1, and LPS in the neuronal (CHP-126) (**A**) and glial (U251) cells (**B**). The bar graph represents band intensity as determined by laser densitometry. Cells were stimulated for the indicated time points before cells were lysed and immunoprecipitated with anti-p65 Ab. The lane labeled "No extract" represents a condition where the cell lysate was omitted. The Western blot was probed with anti-IκBα Ab. Note IκBα degradation in response to TNFα but not MV, IL-1, or LPS in the CHP-126 cells. In contrast, time-dependant IκBα degradation in response to IL-1 and LPS was observed in the glial cell line U251.

the IMR-32 neuronal cells. It is important to emphasize that neuronal cells express the IL-1 receptor (Hart *et al*, 1993). To ascertain that these findings are not unique to the neuronal cell line IMR-32, we examined our findings in an additional cell neuronal cell line CHP-126 (Figure 3). Although MV, TNFα, IL-1, and LPS all resulted in IκBα degradation in the glial cell line U251, degradation in the CHP-126 cells was observed only in response to TNFα. The finding that NF-κB activation in neuronal cells occurred in response to TNFα but not MV, IL-1, or LPS is a key to kinases and factors that could potentially be involved in IκBα phosphorylation. Both IL-1 and LPS use overlapping signaling pathways that result in IκBα phosphorylation (Nasuhara *et al*, 1999; Drupraz *et al*, 2000). Therefore, it is possible that there is "crosstalk" between MV and IL-1 signaling of IκBα, and that a common defect may exist in their signaling pathways in neuronal cells. As indicated earlier, activation of IκBα by TNFα is intact in neuronal cells. Recently, it has become apparent that the signaling pathways for IL-1 and TNFα diverge upstream of the IKK complex (Nasuhara *et al*, 1999; Drupraz *et al*, 2000; Ninomiya-Tsuji *et al*, 1999; Takaesu *et al*, 2001; Li *et al*, 2000; Ozes *et al*, 1999). Therefore, it would appear that a common pathway for MV-, LPS-, and IL-1–initiated responses is defective in neuronal cells, the elements of which remain to be investigated.

Failure of MV to activate NF-$_\kappa$B in neuronal cells is corrected by genetic complementation

To test the hypothesis that neuronal cells are deficient in factor(s) required for IκBα phosphorylation and NF-κB activation, we postulated that genetic complementation with a human brain cDNA library may restore responsiveness to MV, as has been described in other systems (Yamaoka *et al*, 1998). In order to measure NF-κB activation in complemented neuronal cells in response to MV stimulation, neuronal cells were stably transfected with the NF-κB–driven green fluorescent protein (GFP) expression vector SV40 GFP-neo (kindly provided by Drs. Adrian Ting, Mount Sinai School of Medicine, New York, and Brian Seed, Massachusetts General Hospital,

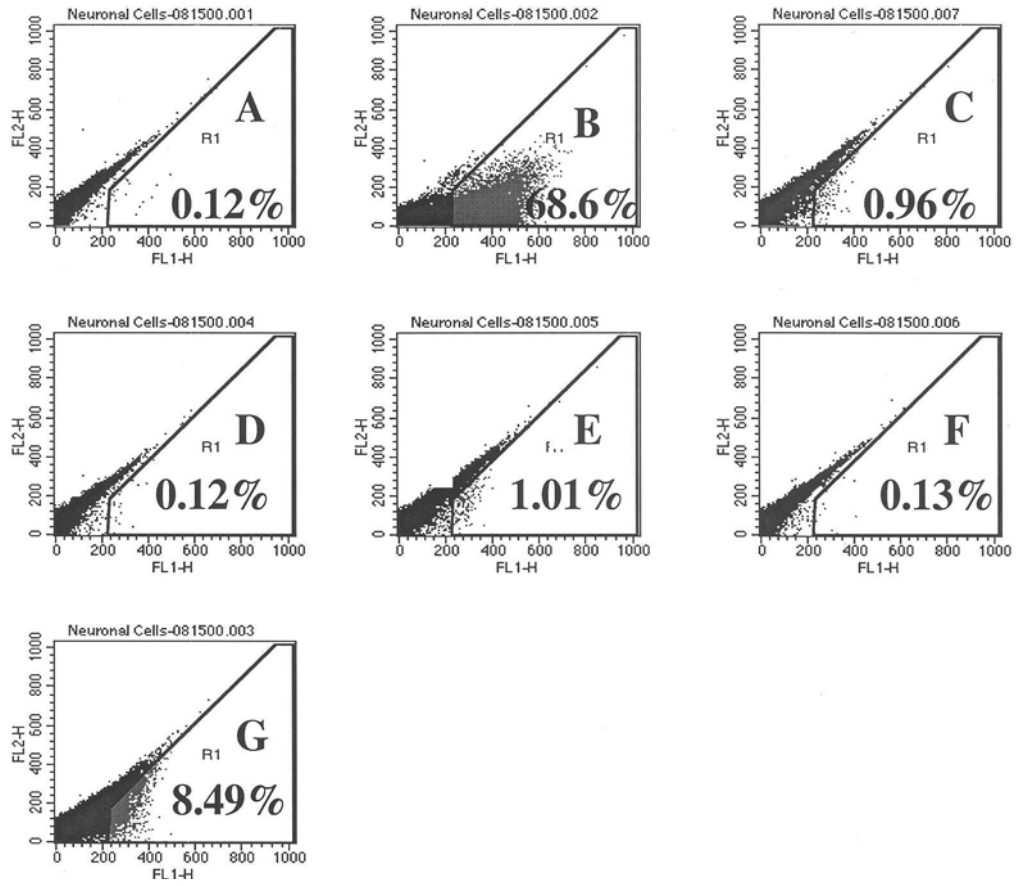

Figure 4 Inducible NF-κB–GFP expression in the IMR-32 neuronal cell clone 3ffe. Cells were transduced with the cDNA library and cultured for 48 h followed by stimulation with 500 U/mL TNFα (as a positive control) or MV (moi = 10) for 4 h. Cells were cultured for an additional 16 h before they were examined for GFP expression by flow-cytometry. The *x*-axis indicates GFP expression. The *y*-axis indicates autofluorescence. (**A**) Unstimulated cells; (**B**) TNFα stimulated; (**C**) MV stimulated; (**D**) empty vector (without cDNA library) transduced cells; (**E**) MV + empty vector; (**F**) cells transduced with vector containing cDNA library; (**G**) cells transduced with vector containing cDNA library then stimulated with MV.

Boston) (Ting *et al*, 1996) using the GenePORTER (Boston, MA) transfection kit as recommended by the supplier (Gene Therapy Systems, CA). GFP expression was analyzed by flow-cytometry following a 4-h stimulation with TNFα or MV and an additional 16-h culture in media without stimulation. The transfected cells were screened by their ability to express high levels of GFP in response to TNFα. Cell clones that exhibited very low background fluorescence and strongly induced GFP in response to TNFα but not MV infection were identified after plating the cells by limiting dilution. One such cloned neuronal cell line stably transfected with κB-GFP (3ffe) was then transduced with a brain cDNA library according to the manufacturer's protocol (Clontech, catalog no. HL8006BB). As shown in Figure 4, neuronal cells transduced with this library (2.4×10^6 IU/ml; multiplicity of infection = 10) can now activate NF-κB in response to MV stimulation. Approximately 8.5% of the library-transduced cells expressed GFP in response to MV, compared to <1% in the absence of MV stimulation. NF-κB was not activated with the "empty vector" or in transduced cells without MV stimulation, indicating that NF-κB activation was MV specific. The results of three separate experiments showed that 5% to 8.5% of the cells were complemented. These results indicate that failure to activate neuronal NF-κB by MV was likely to be due to a deficiency in signaling factor(s) important for NF-κB activation by virus, which remains to be elucidated.

Conclusion

Our results indicate that the IFNβ gene is suppressed in MV-infected neuronal cells, due to lack of activation of a signaling pathway required for NF-κB activation. Although this signaling pathway is not defined at present, it is certainly distinct from that for TNFα and may overlap with those for IL-1 and LPS. These findings have implications on the ability of MV, and perhaps other viruses, to persist in neurons, because IFNβ is a critical host defense mechanism against viral infection.

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