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Mechanism of measles virus failure to activate NF-κB in neuronal cells

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Lack of IFN-β and MHC class I expression in measles virus (MV) infected neurons could impair the host antiviral defense mechanism and result in virus escape from recognition by cytotoxic T-cells. Induction of IFN-β and MHC class I gene expression requires NF-κB activation which depends on degradation of IκBα, an inhibitory protein of NF-κB. In earlier studies we demonstrated that in contrast to glial cells, MV was unable to induce IκBα degradation in neuronal cells. It is unclear whether this failure is due to the presence of a neuron-specific IκBα isoform or a defect in the MV signaling cascade that leads to IκBα phosphorylation and degradation. In this study, an IκBα-wild type (WT) expression vector was transfected into neuronal and glial cells and subsequently exposed to MV. In contrast to glial cells, IκBα-WT was degraded in neuronal cells in response to TNFα but not MV. The findings eliminate the existence of an IκBα isoform in neuronal cells that is resistant to phosphorylation by MV. Blocking *de novo* protein synthesis with cyclohexamide had no effect on neuronal IκBα, indicating that lack of degradation rather than increased synthesis is responsible for IκBα accumulation in MV-stimulated neuronal cells. To determine if malfunction in the MV receptor CD46 is responsible for failure of IκBα phosphorylation and degradation, neuronal cells were transfected with a wild type CD46 (CD46-WT) expression vector. MV stimulation of CD46-WT transfected cells failed to induce IκBα degradation. Collectively these findings indicate that failure of MV to phosphorylate neuronal IκBα is not due to a presence of an IκBα isoform or malfunction of the MV receptor, and is more likely to be due to a defect in the signaling pathway that normally leads to IκBα phosphorylation and degradation. *Journal of NeuroVirology* (2001) **7**, 25–34.

Keywords: measles virus; NF-κB; IκBα; neuronal cells

Introduction

Viral persistence in the central nervous system (CNS) may be caused in part by lower expression of major histocompatibility complex (MHC) antigens on neurons, which allows infected cells to escape recognition by MHC class I-restricted cytotoxic T lymphocytes (CTL) (Joly *et al*, 1991; Oldstone, 1989), the presence of the blood–brain barrier that restricts entry of cells and proteins, and the post-mitotic nature of the neuronal cell population. MV is a neurotropic virus and although self-limiting, infection of the CNS could lead to MV persistence in the form of subacute sclerosing panencephalitis (SSPE) (McFarland and Dhib-Jalbut 1988), and subacute measles encephalitis (SME) in immuno-compromised patients (Dhib-Jalbut and Johnson, 1994). Studies in SSPE and the animal model SME demonstrated that the expression of MHC class I molecules is low on infected neurons (Gogate *et al*, 1996). This is supported by Ward and Massa (1995), who showed that neither virus nor dsRNA was able to induce MHC class I in neonatal mouse neurons. This is in contrast to the situation in glial cells, in which a number of viruses, including murine coronavirus (Suzumura *et al*, 1986), mouse hepatitis virus (MHV-59) (Lavi *et al*, 1989), flavivirus (Liu *et al*, 1989), sindbis virus (Griffin *et al*, 1992), and MV (Kraus *et al*, 1992), are able to induce high expression of MHC class I molecules.

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Moreover, with MV infection, up-regulation of MHC class I in glial cells is mediated by interferon-β (IFN-β) (Dhib-Jalbut and Cowan, 1993). In contrast, MV fails to induce IFN-β expression in neuronal cells (Dhib-Jalbut and Cowan, 1993; Dhib-Jalbut *et al*, 1995). The reason for this failure is lack of NF-κB activation and binding to the positive regulatory domain II (PRDII) element of the IFN-β promoter (Dhib-Jalbut *et al*, 1995) which is necessary for virus inducibility of the IFN-β gene promoter activity (Maniatis *et al*, 1992).

NF-κB is normally sequestered in the cytoplasm by its inhibitor, IκB. Upon stimulation with a variety of agents, including cytokines, virus and dsRNA, IκBα is phosphorylated and degraded through the 26S proteasome complex thereby leading to NF-κB dissociation and translocation from the cytoplasm into the nucleus (Brown *et al*, 1995; Finco and Baldwin 1995; Kumar *et al*, 1994; Li and Sedivy, 1993; Shirakawa and Mizel, 1989). Our previous investigations indicated that MV is able to induce IκBα phosphorylation and degradation in glial cells but not in neuronal cells (Dhib-Jalbut *et al*, 1999). It is not clear whether this failure is due to the existence of a neuron-specific IκBα isoform that is resistant to phosphorylation by MV stimulation or due to a defect in the signaling pathway that leads to IκBα phosphorylation.

The possibility that a neuronal IκBα isoform may exist is inferred from the fact that isoforms of enzymes and transcription factors are often present in different kingdoms from virus (Liu *et al*, 1997) to human (Cao *et al*, 1997). These isoform mRNAs are possibly generated by alternative splicing (Cao *et al*, 1997) or differential usage of the gene promoters (Yasumoto *et al*, 1998). Although these isoforms physically share some exons, functionally they may have distinct transcriptional properties (O'Donovan and Baraban, 1999). Some isoforms may enhance their activity (Nibbs *et al*, 1999) or called activator isoform (Yin *et al*, 1995) while others may play a role of a negative regulator (Ohkura *et al*, 1999) or repressor isoform (Walker *et al*, 1998). To determine whether the lack of IκBα phosphorylation in neuronal cells is due to the presence of a neuron-specific IκBα isoform, ectopic wild type IκBα (IκBα-WT) tagged with the FLAG-epitope was transfected into neuronal cells, and its phosphorylation and degradation patterns in response to MV stimulation were compared with those of native IκBα.

The CD46 or membrane cofactor protein (MCP) has been identified to be functionally associated with susceptibility of cells to MV infection and serves as a cellular receptor for some strains of MV, including Hallé and Edmonston (Dörig *et al*, 1993; Naniche *et al*, 1993a). As a member of the regulators of complement activation (RCA) protein family (Liszewski and Atkinson, 1992), CD46 is ubiquitously present on primate cells and inhibits lysis of host cells by binding the complement cascade components C3b and C4b, and by acting as a cofactor for their proteolytic inactivation by serine protease complement factor I (Liszewski *et al*, 1991). Following infection with MV, CD46 is rapidly down-regulated from the surface of the host cell (Naniche *et al*, 1993b). Studies in five SSPE cases (Ogata *et al*, 1997) showed that CD46 was not detected or was expressed at very low levels in neural cells at the lesion site, whereas normal levels of CD46 were found in SSPE brain tissue distant from the lesion. Furthermore, CD46 was expressed at relatively low levels by neurons and astrocytes in normal brains in comparison to neuroblastoma cell lines (Ogata *et al*, 1997). Little is known about the correlation between CD46 levels and IκBα activation in neuronal cells. Therefore, the possibility that MV failure to trigger IκBα activation in the neuronal cells is due to a CD46 malfunction needed to be addressed.

Results

Expression of IκBα-WT in glial cell

To distinguish ectopic IκBα-wild type from native IκBα, FLAG tagged IκBα-WT was expressed in U-251MG by transient transfection and immunoprecipitated by anti-FLAG M2 antibody while native IκBα was immunoprecipitated by anti-IκBα. As expected, an approximately 37 kDa band corresponding to the native IκBα was immunoprecipitated by anti-IκBα antibody in untransfected (Figure 1, lane 7) and transfected cells (lane 3). Another band of approximately 44 kDa, which corresponded

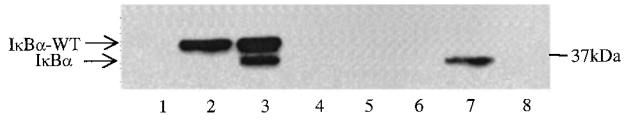

Figure 1 Immunoprecipitation of transfected IκBα-WT from the glial cell line U-251MG. U-251MG cells transfected with epitope-tagged derivatives of IκBα cDNA (IκBα-WT, 20 μg) are shown in the four left lanes and untransfected cells are shown in the four right lanes. Ectopic IκBα-WT could be immunoprecipitated by either monoclonal anti-FLAG or polyclonal anti-IκBα, while native IκBα in transfected and untransfected cells was immunoprecipitated by anti-IκBα antibody only. Immunoprecipitates were fractionated by SDS–PAGE, and analyzed by Western blotting using anti-IκBα antibody as a probe. Immunoprecipitation with normal mouse serum (NMS) or normal rabbit serum (NRS) were used as negative controls. Rainbow™ colored protein molecular weight markers (Amersham), with size covered between 14.3–220 kDa, were used to estimate the molecular weight of the immunoprecipitated bands.

to IκBα-WT, was immunoprecipitated by anti-IκBα from transfected (lane 3) but not untranslated cells (lane 7). In contrast, the anti-FLAG antibody immunoprecipitated a single band in the transfected cells corresponding to the ectopic IκBα (lane 2). These results allowed us to distinguish IκBα-WT from native IκBα.

Phosphorylation and degradation of ectopic IκBα-WT in glial cells

Earlier studies in our laboratory examined the kinetics of IκBα phosphorylation and degradation in response to MV stimulation at time points of 5, 10, 15, 20, 30 min and up to 4 h (Dhib-Jalbut *et al*, 1999). IκBα phosphorylation occurred within 5–10 min of stimulation, and degradation began at 10–15 min later with a half-life of 30 min. This kinetic pattern in glial cells is consistent with that reported in other systems (Finco and Baldwin 1995). Therefore in the following experiment we examined IκBα phosphorylation and degradation at three critical time points: 5, 15, and 30 min following MV-stimulation.

In untransfected U-251MG cells stimulated with TNFα, native IκBα was phosphorylated within 5 min and completely degraded at 15 min. At 30 min, IκBα was regenerated which is consistent with our previous observations (Dhib-Jalbut *et al*, 1999). With MV stimulation, native IκBα phosphorylation occurred as early as 5 min and was significantly degraded at 15 and 30 min (Figure 2A). U-251MG cells transfected with IκBα-WT expression vector expressed ectopic IκBα-WT (Figure 2B). Following stimulation with TNFα, IκBα-WT hyperphosphorylation appeared within 5 min and the band corresponding to IκBα-WT became gradually faint between 15 and 30 min, indicating IκBα degradation. Following MV stimulation, IκBα-WT was phosphorylated after 15 min and degraded after 30 min (Figure 2B). The effect of TNFα and MV stimulation on IκBα-WT degradation in U-251MG cells from two experiments are shown in Figure 2C. The results indicated that, like native IκBα, IκBα-WT responds to TNFα and MV stimulation although the time course of its phosphorylation and degradation was slightly different.

Lack of degradation of IκBα-WT in neuronal cells

Previous studies in our laboratory showed that neuronal IκBα is not hyperphosphorylated nor degraded with MV stimulation at time point of 5, 10, 15, 20, 25, 30, 60 and 120 min although IκBα phosphorylation and degradation occurred in response to TNFα stimulation (Dhib-Jalbut *et al*, 1999). To determine whether this failure is due to the existence of a neuronal IκBα isoform, FLAG-tagged IκBα-WT was transfected into the neuronal cells, IMR-32. IκBα phosphorylation and degradation was examined at 5, 15, and 30 min following TNFα or MV stimulation in untransfected (Figure 3A) and IκBα-WT transfected (Figure 3B) neuronal cells. While TNFα stimulation resulted in IκBα-WT degradation, this was not observed in response to MV (Figure 3A,B). On the contrary, following MV stimulation, the IκBα-WT band became denser

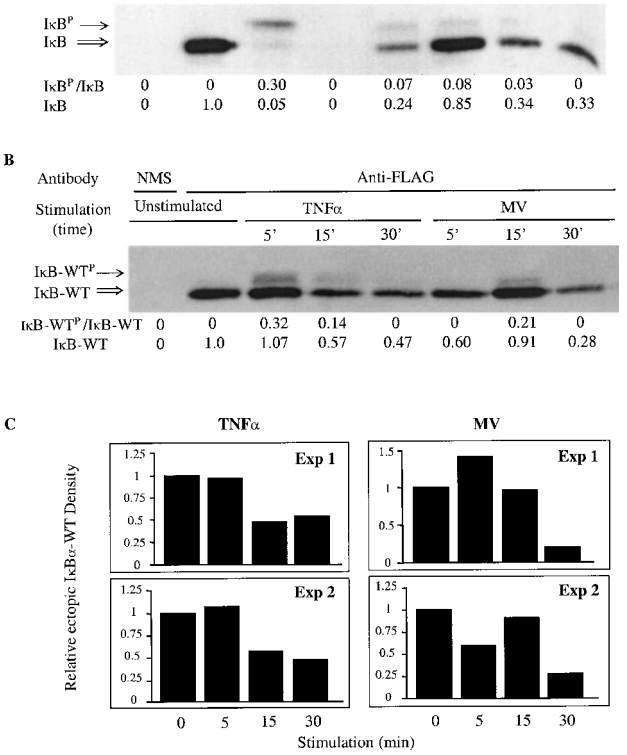

Figure 2 Phosphorylation and degradation of native and ectopic IκBα in response to TNFα and MV stimulation in the glial cell line U-251MG. (**A**) Untransfected U-251MG cells were stimulated by either TNFα or MV and native IκBα from these cells was immunoprecipitated by polyclonal anti-IκBα. The blots were subsequently probed by anti-IκBα antibody. Immunoprecipitation with normal rabbit serum (NRS) was used as a negative control. The upper and lower bands represent the phosphorylated native IκBα (single arrow) and hypophosphorylated native IκBα (double arrow), respectively. (**B**) U-251MG cells were transfected with FLAG epitope-tagged IκBα-WT and stimulated by either TNFα or MV for various time periods. Cytoplasmic extracts were immunoprecipitated with monoclonal anti-FLAG antibody, fractionated by SDS–PAGE, and analyzed by Western blotting using anti-IκBα antibody. The upper bands represent the phosphorylated ectopic IκBα-WT (single arrow) and the lower bands represent the hypophosphorylated ectopic IκBα-WT (double arrow). Normal mouse serum (NMS) was used as a negative antibody control in the immunoprecipitation. The numbers at the bottom of each lane in this and in subsequent figures represent the ratio of the density of phosphorylated to hypophosphorylated IκB ($IκB^P/IκB$) (first row of numbers). The second row represents the ratio of the density of the IκBα band in the treated condition to that in the untreated cells. Zero indicates an undetectable band. (**C**) Summary of the effect of stimulation with TNFα or MV on ectopic IκBα-WT expression in the glial cell line U-251MG obtained from two experiments.

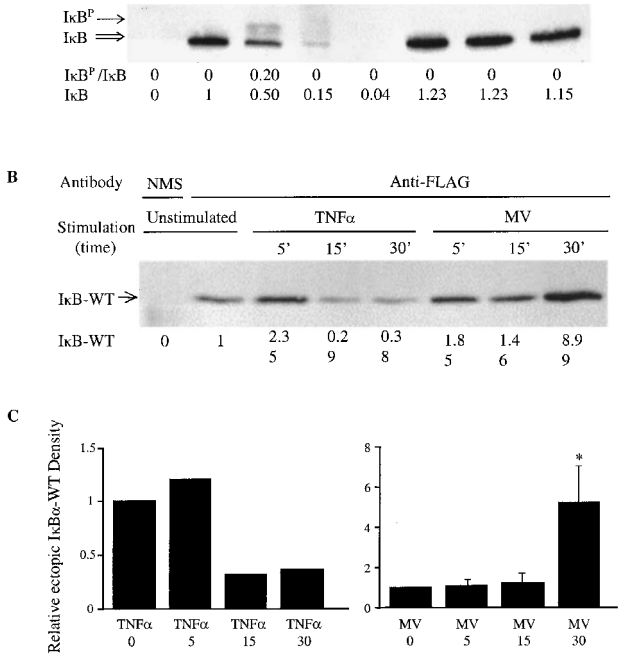

Figure 3 Western blot analysis of native IκBα and ectopic IκBα-WT in untransfected and transfected neuronal IMR-32 cells. (**A**) TNFα but not MV stimulation resulted in phosphorylation and degradation of native IκBα. Native IκBα was immunoprecipitated with polyclonal anti-IκBα, fractionated by SDS–PAGE, and probed with anti-IκBα. Phosphorylation was observed after 5 min of TNFα stimulation and degradation was apparent after 15 min. As expected with MV stimulation, neither phosphorylation nor degradation of native IκBα occurred. (**B**) Ectopic IκBα-WT was immunoprecipitated with monoclonal anti-FLAG antibody, fractionated by SDS–PAGE, and probed with anti-IκBα antibody. Ectopic IκBα-WT was degraded after 15 min of TNFα stimulation. In contrast, the bands of ectopic IκBα became denser following MV stimulation. (**C**) Summary of the effect of stimulation with either TNFα or MV on ectopic IκBα-WT expression in the neuronal cell line IMR-32. The figure shows average from four experiments. Error bars depict standard errors of the mean normalized density derived from four independent transfections. Asterisk indicates a significant difference ($P<0.05$) between MV stimulation for 30 min and untreated control.

(Figure 3B). To quantitate this result, we averaged the effect of MV stimulation on IκBα-WT in transfected neuronal cells in four separate experiments (Figure 3C). We found that IκBα-WT level was significantly increased ($P<0.05$) after 30 min of MV stimulation, compared to the control condition. This indicated that IκBα-WT was not degraded but its expression was rather increased by MV stimulation. Thus, it is unlikely that lack of IκBα degradation in MV-stimulated neuronal cells is due to a 'mutant' IκBα isoform.

We have observed that the immunoprecipitated band corresponding to the transfected IκBα-WT was denser at 30 min following MV stimulation. Theoretically this could represent an artifact of the system used (i.e. MV stimulation of the CMV promoter in the IκBα-WT construct), or that MV stimulation leads to enhanced synthesis and accumulation of IκBα. Therefore to determine whether the increased IκBα-WT was due to MV induction of the CMV promoter, we transfected neuronal cells with a reporter gene (β-gal) under the control of the CMV promoter (pCMV β-gal) examined β-galactosidase activity in response to MV stimulation using an *in situ* β-galactosidase staining kit (Clontech, CA, USA). Forty-eight hours after transient transfection with pCMV β-gal, the cells were stimulated with MV for 5, 15 or 30 min. Transfected cells without MV stimulation were used as controls. The cells were then stained *in situ* according to the supplier's instruction. The images of stimulated and unstimulated cells were captured using a CCD camera at randomly selected fields. The average results from three experiments are shown in Figure 4. The results indicate that MV is capable of stimulating the CMV promoter after 30 min of stimulation. Therefore the increase in IκBα-WT level was most likely driven by an effect of MV on the CMV promoter, rather than an effect on IκBα synthesis and accumulation.

Effects of MV stimulation on neuronal IκBα synthesis and accumulation

To determine whether there is lack of neuronal IκBα degradation versus enhanced IκBα synthesis in response to MV, we examined the effect of blocking *de novo* protein synthesis with cyclohexamide on IκBα levels in MV stimulated neuronal cells. Cells were pretreated with cyclohexamide (Sigma, MO, USA) (100 μg/ml) for 1 h prior to MV or TNFα stimulation. Cyclohexamide-untreated neuronal cultures served as controls. TNFα stimulation was used as a control since it induces IκBα degradation in neuronal cells. The results are shown in Figure 5. As expected, in cyclohexamide-untreated cells, IκBα degradation was observed in response to TNFα but

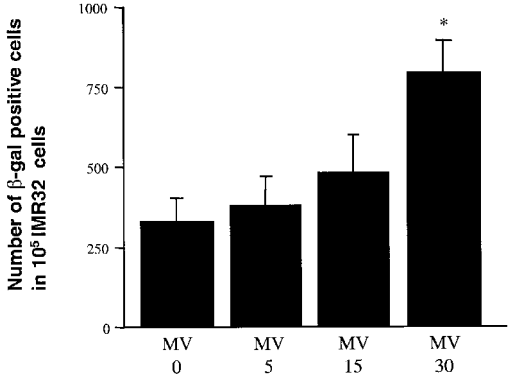

Figure 4 β-galactosidase activity in pCMV β-gal transfected neuronal cells in response to MV stimulation at several time points. The graph represents means from three experiments. The asterisk indicates a P value <0.01.

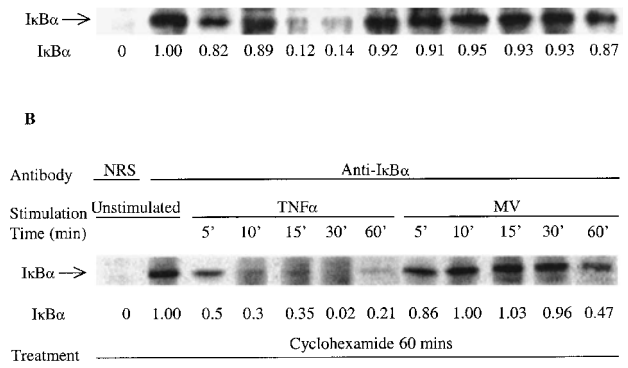

Figure 5 Effect of *de novo* protein synthesis blockade with cyclohexamide on neuronal IκBα band density in response to MV or TNFα stimulation (as a positive control) for time periods ranging from 5 to 60 min. (**A**) without and (**B**) with cyclohexamide. The numbers at the bottom indicate the densitometric measurements of the IκBα band relative to the band density in the unstimulated lane.

not MV stimulation at time periods ranging from 5 to 60 min (Figure 5A). In cyclohexamide treated cells (Figure 5B), IκBα degradation was observed in response to TNFα within 5–10 min post stimulation. In contrast to the cyclohexamide-untreated cells, there was no regeneration of the IκBα band after 60 min of TNFα stimulation, indicating that cyclohexamide effectively blocked *de novo* IκBα synthesis. Importantly, no change in the density of the IκBα band was observed up to 30 min post MV stimulation in cyclohexamide pre-treated cells. This indicates that the unchanged IκBα band density is due to lack of degradation rather than increased synthesis and accumulation of IκBα in response to MV. At 60 min post MV stimulation, the density of the IκBα band was reduced by approximately 50%, possibly reflecting autolysis rather than signal induced degradation. The intensity of this IκBα band at 60 min time point was 40% less in the presence of cyclohexamide, compared to the same time point in the absence of cyclohexamide, again reflecting the fact that cyclohexamide was effective in blocking *de novo* IκBα synthesis. Collectively, these results support the notion that there is lack of degradation of neuronal IκBα in response to MV stimulation.

Effect of CD46-WT expression in the neuronal cell line on IκBα phosphorylation and degradation

To determine if engagement of the MV receptor in the absence of infection is sufficient to signal NF-κB activation, we had previously examined the effect of UV-inactivated MV on NF-κB DNA binding activity and IκBα degradation (Dhib-Jalbut *et al*, 1999). Both live and UV-inactivated virus resulted in IκBα degradation and NF-κB activation, suggesting that binding of the virus to its receptor is sufficient for signaling the IκBα/NF-κB system. Therefore, we next examined the possibility that lack of IκBα degradation in neuronal cells may involve a MV receptor defect. To determine if lack of IκBα phosphorylation and degradation in neuronal cells is due to a MV receptor (CD46) defect, IMR-32 cells were transfected with a CD46 expression vector. Both transfected and untransfected IMR-32 cells were stained with FITC-conjugated mouse anti-CD46 or FITC-conjugated mouse control IgG isotype and examined by flow cytometry (Figure 6). Compared with untransfected cells, CD46 was expressed at higher levels in transfected cells (Δ mean fluorescence density of 40 for transfected cells compared to 26 for untransfected cells). IMR-32 cells transfected with CD46-WT were stimulated with TNFα or MV and analyzed by Western blot to identify whether failure of IκBα degradation was due to a CD46 malfunction. IκBα phosphorylation and degradation were examined at 10, 30, and 60 min post MV stimulation which represent focal time points for IκBα phosphorylation, degradation, and regeneration respectively. A representative of two experiments is shown in Figure 7. Phosphorylation and degradation of IκBα in transfected neuronal cells was not observed after MV stimulation although IκBα was phosphorylated and degraded in response to TNFα stimulation. Therefore, the failure of phosphorylation and degradation of IκBα following MV stimulation is not due to an abnormality involving the MV receptor on neuronal cells.

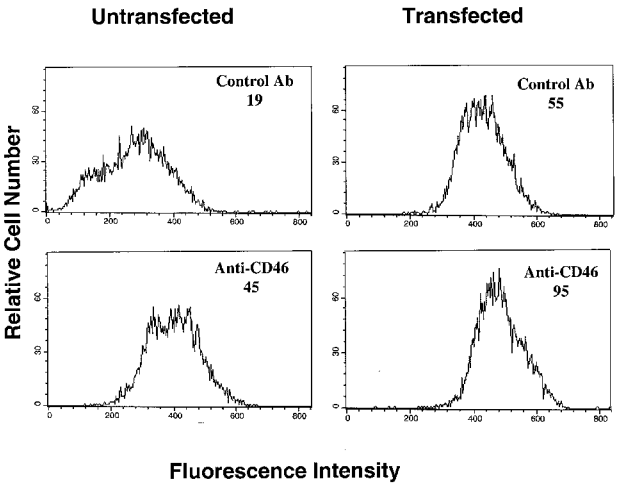

Figure 6 Expression of CD46-WT in transfected neuronal cells. Both untransfected and transfected neuronal cells were stained with FITC-conjugated mouse control IgG (upper panel) and FITC-conjugated mouse anti-CD46 (lower panel). The numbers within each box represent mean fluorescence intensity. The difference in mean fluorescence intensity between the experimental and control antibody was 40 for transfected cells compared to 26 for untransfected cells.

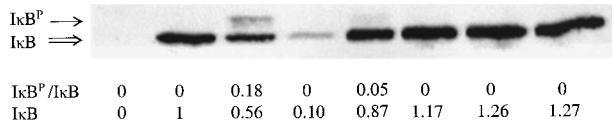

Figure 7 Kinetics of IκBα phosphorylation and degradation in CD46-WT transfected neuronal cell line IMR-32 in response to TNFα and MV stimulation. IMR-32 cells were transfected with CD46-WT and stimulated with TNFα or MV for the indicated time periods. IκBα was then immunoprecipitated with polyclonal anti-IκBα, fractionated by SDS–PAGE, and probed with anti-IκBα. IκBα phosphorylation was observed after 10 min of TNFα stimulation and degradation was apparent after 30 min. After 1 h stimulation, IκBα was regenerated. With MV stimulation, neither phosphorylation nor degradation of native IκBα occurred in CD46-WT transfected IMR-32.

Discussion

The expression of MHC class I on the surface of infected cells is a key step for recognition of viral antigens by immune surveillance with $CD8^+$CTL (Oldstone, 1989; Dhib-Jalbut *et al*, 1990). Previous studies from our laboratory demonstrated that infected neurons from human autopsies of SSPE and in the animal model SME express low levels of MHC class I molecules (Gogate *et al*, 1996). We also found that the expression of MHC class I is enhanced by IFN-β in glial cells, whereas both molecules are not expressed on neuronal cells in response to MV (Dhib-Jalbut *et al*, 1993, 1995). NF-κB is a transcription factor required for both IFN-β and MHC class I induction (Dhib-Jalbut *et al*, 1993). It is retained in the cytoplasm through interaction with inhibitory proteins including IκBα. Exposure of cells to stimuli such as TNFα, results in the phosphorylation and degradation of IκBα. Dissociated NF-κB dimers are then translocated to the nucleus, where they bind to the positive regulatory domain (PRD) II of the IFN-β promoter and the IFN-β gene is transcribed.

In an earlier study, we demonstrated that in contrast to glial cells, MV was unable to induce IκBα phosphorylation and degradation in neuronal cells (Dhib-Jalbut *et al*, 1999). In this study we investigated whether this failure is due to the presence of a neuron-specific IκBα isoform. The results demonstrate that untransfected glial cells as well as those transfected with IκBα-WT responded to MV stimulation, resulting in IκBα-WT phosphorylation and degradation. Under the same conditions, neither phosphorylation nor degradation of IκBα-WT occurred in response to MV in neuronal cells transfected with IκBα-WT. This was also true of another neuronal cell line, CHP 126 (unpublished observations). However, following TNFα stimulation, IκBα degradation occurred in both transfected and untransfected cells from both glial and neuronal cell lines. Based on these observations, we conclude that failure of MV stimulation to result in IκBα phosphorylation and degradation in neuronal cells is unlikely to be due to the existence of a neuron-specific IκBα isoform.

We also explored the possibility that the unchanged IκBα band density following MV stimulation might be due to enhanced synthesis rather than lack of degradation. Blocking *de novo* protein synthesis with cyclohexamide had no effect on IκBα band density within the expected time for its degradation in response to MV stimulation. This suggests that lack of degradation rather than enhanced synthesis is responsible for the accumulation of IκBα in MV stimulated neuronal cells.

Membrane cofactor protein (MCP, CD46) is a widely distributed regulatory protein of the complement system that facilitates inactivation of C3b/C4b (Seya *et al*, 1986, 1990). It consists of six unique mRNA isoforms with three different STP (serine/threonine/proline) rich regions and two distinct cytoplasmic tails due to alternative splicing (Post *et al*, 1991). Lately it was identified as MV receptor by binding MV hemagglutinin (HA) protein at two distinct regions of the CD46 complement control protein domains (CCP domain 1 and CCP domain 2) (Dörig *et al*, 1993; Naniche *et al*, 1993a; Buchholz *et al*, 1997). Our study indicates that UV-inactivated MV is capable of inducing IκBα degradation and NF-κB activation. This suggests that ligation of the MV receptor is sufficient to signal the IκBα/NF-κB system, without the need for infectious virus. Although little is known about the pathway from CD46 stimulation to IκBα phosphorylation, a mutation in CD46 on neuronal cells may contribute to failure of IκBα activation. To address this hypothesis, we transfected CD46-WT into neuronal cells and stimulated those cells with MV. IκBα phosphorylation and degradation did not occur in those cells despite enhanced CD46 expression. These results suggest that a defect in the signaling cascade distal to the MV receptor complex, may contribute to the failure of IκBα phosphorylation in neuronal cells in response to MV. However, we cannot exclude the possibility that MV signaling is occurring via another receptor. Using commercially available antibodies to CD46, we have not been able to block MV infection of neuronal cells as determined by cell surface MV protein expression (data not shown).

Many agents can activate NF-κB, including cytokines, double-stranded RNA, virus, and TNFα (Kumar *et al*, 1994; Dhib-Jalbut *et al*, 1995; Malinin *et al*, 1997). It is still unclear whether different stimuli utilize independent signaling pathways, or share a common pathway that leads to IκBα phosphorylation. Several kinases have been shown to be involved in IκB phosphorylation, including

the double-stranded RNA-dependent protein kinase (PKR) (Maniatis *et al*, 1992), PKC and PKA (Shirakawa and Mizel, 1989), Raf-1 (Li and Sedivy, 1993), casein kinase II (CKII) (Barroga *et al*, 1995), and IKK (Malinin *et al*, 1997; Maniatis, 1997). PKR is a serine-threonine kinase involved in growth inhibition and NF-κB activation through phosphorylation of eIF2a and IκBα respectively (Clemens and Elia, 1997). PKR activation requires dsRNA binding to the domain at its N-terminal, auto-phosphorylation and dimerization. Studies in PKR knockout mice demonstrated a lack of NF-κB activation by poly (I)·poly (C), but this activation remained intact in response to TNFα (Yang *et al*, 1995). To exclude the possibility of a PKR defect in the inability of MV to induce IκBα degradation in neuronal cells, we have previously performed PKR auto-phosphorylation assays in MV stimulated neuronal and glial cells. The results showed that MV was unable to phosphorylate IκBα in neuronal cells despite PKR activation (Dhib-Jalbut *et al*, 1999), suggesting either a failure to activate a signal distal to PKR in the signaling cascade that leads to IκBα phosphorylation or the existence of a PKR-independent pathway used by virus to phosphorylate IκBα (Dhib-Jalbut *et al*, 1999).

At present, the signaling pathway for MV-induced IκBα phosphorylation is not understood. A pathway of TNFα induced IκBα phosphorylation by an IκBα kinase complex (IKK) has been described (Malinin *et al*, 1997; Maniatis, 1997). This kinase complex consists of two catalytic subunits: IKKα and IKKβ (DiDonato *et al*, 1997; Mercurio *et al*, 1997) and a regulatory subunit IKKγ (Rothwarf *et al*, 1998). IKKβ is phosphorylated by mitogen-activated protein kinase/ERK kinase kinase-1 (MEKK1) (Nakano *et al*, 1998), whereas IKKα is phosphorylated by NF-κB-inducing kinase (NIK) (Malinin *et al*, 1997). In addition to the NIK-IKK pathway, a recent study demonstrated the involvement of PI(3)K-Akt pathway in IκBα phosphorylation in response to TNFα stimulation (Ozes *et al*, 1999). We are currently investigating whether the NIK-IKK and PI(3)K-Akt pathways are involved in MV-induced IκBα phosphorylation in glial cells. If so, a defect in one of these pathways could be responsible for lack of IκBα phosphorylation in response to MV in neuronal cells.

In summary, this study demonstrates that failure of MV to phosphorylate and degrade IκBα in neuronal cells is not due to a neuron-specific IκBα isoform nor an abnormal MV receptor. A defect in the signaling cascade distal to the MV receptor is likely, and is currently being investigated.

Materials and methods

Plasmids preparation

The plasmid expressing wild type IκBα (IκBα-WT) tagged with FLAG-epitope was kindly provided by Dr Dean W Ballard (Vanderbilt University, Tennessee, USA). Epitope-tagged derivatives of cDNA encoding full length IκBα-WT (Haskill *et al*, 1991) were constructed by PCR-assisted amplification with 5′ primers that fused sequences encoding the FLAG epitope (Prickett *et al*, 1989) in frame with N-terminal coding sequences of IκBα-WT (5′-CCCAAGCTTCCACCATGGACTACAAAGACGAT-GACGATAAAATGTTCCAGGCGGCCGAGCGC-3′). This construct was cloned into the *Hind*III and *Xba*I polylinker site of the eukaryotic expression vector pCMV4 (Andersson *et al*, 1989) immediately downstream of the cytomegalovirus immediate-early promoter and purified with QIA filter plasmid kit (QIAGEN). The reported molecular weight of IκBα-WT is 44 kDa (Brockman *et al*, 1995).

MCP-BC2, a CD46 cDNA isoform was cloned into a mammalian expression vector (pSG5) (Post *et al*, 1991). This construct was kindly provided by Dr John Atkinson (Washington University Medical School, USA). The plasmid was prepared with QIA filter plasmid kit (QIAGEN).

Cell cultures

The neuronal cell line IMR-32 cell was a gift from Dr Richard J Ziegler (University of Minnesota, Duluth, MN, USA) and was maintained in Eagle's minimum essential medium (EMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 10 mM Hepes buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml gentamycin. The human astrocytoma cell line U-251 MG was kindly provided by Dr Darryl Bigner (Duke University, North Carolina, USA) and grown in Dulbecco's modified Minimal Essential Medium (DMEM) supplemented with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 0.1 mM non-essential amino acids, 1 ×MEM vitamins, 10 mM Hepes buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml gentamycin.

Preparation of measles virus (MV)

The Edmonston strain of MV was obtained from ATCC (Rockville, MD, USA), grown in confluent vero cells (monkey kidney fibroblasts from ATCC), and titrated by plaque assay according to standard methods (Dhib-Jalbut and Cowan, 1993). The stock titer was 5×10^7 plaque forming units/ml.

Transient transfection

IMR-32 (1×10^7) or U-251MG (2×10^5) cells were plated in 100 mm culture dishes and incubated at 37°C in 5% CO_2 for 24 h. U-251MG cells were plated at a lower density than IMR-32 because the former cells divide much faster. IκBα-WT (20 μg) was transiently transfected into either IMR-32 or U-251MG cells using the calcium phosphate precipitation method as recommended by the supplier, profection® mammalian transfection systems (Promega, WI, USA). The cells were then incubated for

6 h with calcium phosphate-DNA co-precipitation, followed by two washes in ice-cold phosphate-buffered saline (PBS). Fresh media was then added, and the cells were incubated for an additional 48 h. Untransfected controls were prepared in parallel with the transfected cells. Both transfected as well as untransfected cells were washed twice with ice-cold PBS before infection with MV (5×10^7 plaque-forming units/ml), or treatment with human recombinant tumor necrosis factor-alpha (TNFα, 150 units/ml) (Genzyme, MA, USA) for 5 to 30 min. In other experiments, CD46-WT was transiently transfected into IMR-32 cells using lipofection method as recommended by the supplier, GenePORTER Transfection Reagent (Gene Therapy Systems, CA, USA). IMR-32 (1×10^7) cells were plated in 100 mm culture dishes and incubated at 37°C in 5% CO_2 for 24 h. The cells were incubated with a mixture of CD46-WT (10 μg) and transfection reagent (50 μl) in 10 ml of serum free EMEM supplemented with 2 mM L-glutamine, 10 mM Hepes buffer, 100 units/ml penicillin, 0.1 mg/ml streptomycin and 0.25 mg/ml gentamycin at 37°C in 5% CO_2 for 4 h. The culture medium was replaced with 20% EMEM and further incubated for 48 h.

Flow cytometry analysis

To monitor CD46-WT transfection efficiency, direct immunofluorescence analysis of the relative expression of neuronal cell surface molecules was determined using FACScan (Becton Dickinson). A total of 2×10^6 transfected or untransfected neuronal cells were washed with phosphate-buffered saline (PBS)/5% fetal calf serum (FCS) and individually incubated with 20 μl of FITC-conjugated mouse anti-CD46 or 1 μg of FITC-conjugated mouse control IgG isotype (Pharmingen, San Diego, USA) for 1 h on ice, in 100 μl PBS/5% FCS at 4°C and fixed with 4% paraformaldehyde in PBS. The cells were then analyzed by flow cytometry and the mean fluorescence intensity of the total cell population was used in the analysis.

Immunoprecipitation and Western blotting

Immunoprecipitation and Western blotting were performed as described earlier (Dhib-Jalbut *et al*, 1999). Briefly, after washing twice in ice-cold PBS, cells were lysed in 1 ml of TNT-E lysis buffer (20 mM Tris, 50 mM NaCl, 1% Triton X100, 5 mM EDTA) containing the following protease inhibitors: 50 mM sodium fluoride, 0.1 mM sodium orthovanadate, 20 mM β-glycerphosphate, 10 mM molybdic acid, 21 μg/ml aprotinin, and 0.2 mM AEBSF (Sigma, MO, USA). Cell lysates were centrifuged at 10 000 ×g for 10 min at 4°C. FLAG epitope-tagged IκBα was immunoprecipitated from the cytosolic extracts of transfected cells, by incubation with 50 μl of agarose beads conjugated to monoclonal anti-FLAG M2 (Sigma, MO, USA) for 16 h at 4°C with constant rocking. Native IκBα was immunoprecipitated from cytosolic extracts of untransfected cells, by incubation with 1 μg of anti-IκBα antibody (amino acids 1–28 and 229–317, Santa Cruz, CA, USA) for 2 h, followed by 50 μl of protein A sepharose (Amersham, AB) for 16 h at 4°C with constant shaking. Immunoprecipitates were washed three times with the above TNT-E buffer containing protease inhibitors, heat denatured in 50 μl of 2 × Laemmli sample buffer (Sigma, MO, USA) for 5 min, fractionated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) at 75 V for 16 h and electrophoretically transferred to a Hybond ECL membrane (Amersham) at 30 V overnight. Rainbow™ colored protein molecular weight markers (Amersham) was used as an index. Membranes were blocked with Tris-buffered saline (DAKO) containing 5% non-fat dry milk for 1 h at room temperature and then incubated with a rabbit antiserum (Sigma, MO, USA) against IκBα (1 μg/ml) for 1 h at room temperature. The blots were washed three times with TBS-milk, then incubated with horseradish peroxidase conjugated donkey anti-rabbit IgG (Amersham, 1 : 10,000 dilution) for 1 h. After three washes with TBS, immunoreactive products were detected using enhanced chemiluminescence system (Amersham).

Statistical analysis

The Western blot films were digitized with a video camera and the density of the bands was determined by a universal software 1D main (Advanced American Biotechnology, Fullerton, CA). Values were quantitatively represented in arbitrary units relative to the density of each band, which were normalized against the density of the IκB band immunoprecipitated from untreated cells. The phosphorylated IκB band ($I\kappa B^P$) density was expressed relative to the density of the hypophosphorylated band ($I\kappa B^P/I\kappa B$) in each lane. The data was expressed as Mean ± SEM and compared using one way ANOVA followed by a post-hoc Dunnett's test.

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