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Interferon-Induced Tetherin Restricts Vesicular Stomatitis Virus Release in Neurons

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Tetherin, a recently identified interferon (IFN)-inducible, type 2 transmembrane protein, has been shown to be a cellular antiviral restriction factor that retains newly formed virions in infected cells. Thus, tetherin plays an important role in the innate cell-autonomous immune response. The aim of this study was to examine the antiviral activities of tetherin in vesicular stomatitis virus infections of murine neuronal cells. Both IFN-β and IFN-γ induce the expression of tetherin mRNA and protein. Tetherin knockdown experiments were carried out by transfection of tethrin shRNA into murine neuroblastoma cells using a vector containing the pCMV-driven *tGFP* gene. The efficiency of transfection was monitored through GFP expression by the transfected cells. Selected transfected cells were used for further mRNA and protein analysis, fluorescent immunocytolocalization, and viral infection to study the impact of tetherin knockdown. Our research indicates that tetherin is expressed on the outer face of the plasma membrane of murine neuroblastoma cells, its expression can be induced with both IFN-γ and IFN-β, and tetherin restricts progeny virus release up to 100-fold in mammalian neurons, thus contributing to a potent antiviral state within the host cell.

Introduction

The critical role interferons (IFNs) play in innate antiviral immunity has been studied in detail (Goodbourn *et al.*, 2000; Levy and Garcia-Sastre, 2001; Samuel, 2001; Pestka *et al.*, 2004). Pathogen recognition molecules, including Toll-like receptors (TLR), retinoic acid-inducible gene-I (RIG-I) -like receptors, and NOD-like receptors, are actively involved in induction of IFNs soon after viruses infect cells (Roberts *et al.*, 2003; van Pesch *et al.*, 2004; Akira *et al.*, 2006; Pichlmair and Reis e Sousa, 2007; Kumagai *et al.*, 2008; Takeuchi and Akira, 2008). Subsequently, IFNs bind their receptor and signal via the JAK-STAT pathway, and induce expression of a variety of antiviral IFN-stimulated genes (ISGs) that are highly critical for antiviral responses (Levy and Darnell, 2002; Haller *et al.*, 2007). Mice deficient in any component of the IFN pathway are more susceptible to lethal viral infection (Hwang *et al.*, 1995; Durbin *et al.*, 1996; Meraz *et al.*, 1996).

Among important ISGs with well-established antiviral activities are RNaseL/2′,5′-oligoadenylate synthase, ISG15, myxovirus resistance (Mx), protein kinase R (PKR), nitric oxide synthase, and RNA-specific adenosine deaminase that inhibit virus infection by different mechanisms (Chesler and Reiss, 2002; Sadler and Williams, 2008; Reiss, 2009; Kuhl *et al.*, 2011). Thus, the replication of viruses in eukaryotic cells is inhibited by a variety of ISGs. An important molecule among these is the recently identified cellular antiviral restriction factor tetherin, which is an IFN-inducible, 28- to 36-kDa transmembrane protein. It has two membrane anchors: a transmembrane domain near the N-terminus and a glycophosphatidylinositol-linked anchor at the C-terminus. This protein is also known as Bst-2 and CD317. The ectodomain of the protein features three cysteine residues that may mediate homodimerization: two N-linked glycosylation sites and a coiled-coil domain (Neil *et al.*, 2008). To restrict release of the newly assembled viral particles from the infected cells, tetherin retains the virions at the cell surface (Schubert *et al.*, 2010). Expression of tetherin is strongly induced by IFN due to the presence of IFN response elements (IREs) and a binding site for STAT3 in the promoter region (Blasius *et al.*, 2006). Additional response elements are present in tetherin promoter consistent with induction by other inflammatory cytokines such as IL-6 and TNF-α (Sauter *et al.*, 2010). The level of tetherin expression appears to be cell-type dependent (Miyagi *et al.*, 2009).

In this study, we have extended our investigations of the mechanisms of IFN-induced antiviral pathways in neurons. We have observed IFN-dependent tetherin upregulation in mouse neuroblastoma cells. We use vesicular stomatitis virus (VSV) infections. A member of *Rhabdoviridae*, order *Mononegavirales*, VSV encodes five major proteins in < 12 kb of RNA: the glycoprotein (GP) G, nucleocapsid protein N, phosphoprotein P, matrix protein M, and large protein L (Rose and Whitt, 2001). VSV replicates readily in IFN pathway-defective cells, but poorly in IFN-responsive normal tissues due to its

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exquisite sensitivity to the antiviral pathways induced by IFNs (Stojdl *et al.*, 2000; Chesler and Reiss, 2002; Trottier *et al.*, 2005; Zhang *et al.*, 2010).

Among the pattern recognition receptor (PRR) families, TLRs are one of the best characterized. They are responsible for sensing the invading pathogens outside the cell, and in intracellular endosomes and lysosomes as well (Akira, 2006). Nucleic acids derived from viruses are recognized by TLR3, TLR7, TLR8, and TLR9. Endosomal TLR7 and TLR 8 recognize ssRNA, and TLR3 is activated by dsRNA from RNA viruses (Akira, 2006; Baum and Garcia-Sastre, 2010). Type I IFNs are produced as a result of a MyD88-dependent pathway (Negishi *et al.*, 2006; Takeuchi and Akira, 2010). RIG-I and melanoma differentiation-associated gene 5 (*MDA5*) are important for cell signaling activated by cytoplasmic RNAs (Takeuchi and Akira, 2008; Wilkins and Gale, 2010). Lack of RIG-I in mouse fibroblasts and cDCs results in defective production of type I IFNs and inflammatory cytokines in response to various RNA viruses, including VSV (Kato *et al.*, 2006). VSV produces dsRNA in infected cells (Kato *et al.*, 2008) and IFN-γ-inducing activity is highly reduced when these dsRNAs are disrupted; to evade RIG-I recognition, the dsRNA particles produced by VSV are much shorter in size than genomic RNA (Takeuchi and Akira, 2010). VSV G is essential for the induction of CD14/TLR4-dependent response pathway, which gives the adapter TRAM prime importance. Downstream of TRAM cascade, IRF7 activation leads to type 1 IFN production (Georgel *et al.*, 2007).

Injection of VSV into immunocompetent mice via many routes results only in the induction of type I IFNs (Trottier *et al.*, 2007; Reiss, 2009), and strong innate and adaptive immune responses with no viral replication or disease. Intranasal inoculation in mice results in infection of the olfactory bulb and retrograde progression in the central nervous system, resulting in acute encephalitis, break down of the blood–brain barrier with high rate of mortality (Olitsky *et al.*, 1933; Huneycutt *et al.*, 1993; Plakhov *et al.*, 1995; Chauhan *et al.*, 2010). Experiments with VSV infections of IFN-β-treated primary neurons and NB41A3 cells are consistent with a highly attenuated infection, associated with production of viral RNA and proteins, dysregulation of viral protein phosphorylation, and impaired viral assembly and release (Trottier *et al.*, 2005; D'Agostino *et al.*, 2009a).

In this study, to understand the role played by tetherin in mammalian neurons, we studied the expression of tetherin in murine neuroblastoma cells. We observed that expression was upregulated with both IFN-γ and IFN-β treatment. We silenced the expression of tetherin in murine neuroblastoma cells using shRNA. Tetherin-silenced cells were characterized and then subjected to VSV infection to compare the difference in viral titers with that of tetherin expressing cells.

Materials and Methods

Cell culture: virus

Murine NB41A3 cells (ATCC) maintained in F12K (Mediatech, Inc.) medium, supplied with 15% horse serum, 2.5% fetal bovine serum, and 1% penicillin/streptomycin (Trottier *et al.*, 2005; D'Agostino and Reiss, 2010). VSV (Indiana serotype, San Juan strain) was the gift from Alice S. Huang (then at The Children's Hospital, Boston). Plaque assay on L929 adipocyte monolayers was used to measure the viral titer of supernatants obtained after VSV infection (Trottier *et al.*, 2007), and was modified with an overlay of Methyl cellulose (Sigma) followed by incubation at 37°C overnight (Robin *et al.*, 1982). Plates were then washed and plaques were counted and viral titer calculated. IFN-β, purchased from Cell Sciences, Inc., was used at 400 U/mL for 24 h treatment, as previously published (Trottier *et al.*, 2005). Mouse IFN-γ (R&D Systems) treatments were done in complete media at 20 ng/mL and incubated at 37°C for 72 h, as previously published (Chesler *et al.*, 2004).

Expression assays

Western blot assays, RNA extraction, RT-PCR, and immunofluorescence were performed as previously published (Ireland *et al.*, 2005; D'Agostino and Reiss, 2010). For mRNA, purified RNA (5 μg) was amplified with tetherin forward and reverse primers by RT-PCR having sequences 5′ TCAGGAGTCCCTGGAGAAGA 3′ and 5′ ATTCTCCAGC TCCTGGTTCA 3′ (100 bp product). β-actin forward primer sequence was 5′ AAGAGCTATGAGCTGCCTGA 3′, and reverse primer sequence was 5′ TACGGATGTCAACGTCAC AC 3′ (200 bp product). Primary antibodies for western blot included rabbit anti-BST2 (1:1,000; for tetherin; Sigma Aldrich), and rabbit anti-β-actin (1:2500; Abcam); and horseradish peroxidase-labeled donkey anti-rabbit IgG (1:2500; Zymax). For confocal microscopy on a Leica TCS SP5, the primary antibodies used were rabbit anti-Bst2 antibody (1: 500) for tetherin, mouse anti-NMDA receptor subunit 1 antibody (1:1000) (Abcam), and Alexa Fluor 633 (1:500; red; Invitrogen) and Alexa Fluor 488 (1:500; green; Invitrogen) as the secondary antibodies. Cells (unfixed) were treated with a primary antibody, and then permabilized and stained with DAPI to identify nuclei (D'Agostino and Reiss, 2010). In western blots and RT-PCR studies, individual bands on scanned films or gels were analyzed using UN-SCAN-IT™ gel software version 5.1.

Transfection with shRNA plasmids

Synthetic shRNA constructs (HuSH plasmid constructs from Origene™) used to silence the expression of tetherin include GFP and puromycin resistance as detection and selection markers, respectively. Turbofectin 8.0 (Invitrogen) solution, shRNA plasmid (100 ng/μL), and serum-free DMEM were mixed together and incubated at room temperature for 45 min and added to murine neuroblastoma cells (3×10^5), in a six-well culture dish followed by incubation of the cells at 37°C for an hour. A control plasmid containing scrambled shRNA (100 ng/μL) was also transfected same as above. After the incubation period, the medium was replaced with complete F12K medium and incubated at 37°C overnight. The medium was then replaced with medium containing puromycin (10 μg/mL). The expression of GFP was monitored by fluorescence microscopy (Olympus BH2; Olympus) to ensure the efficiency of transfection.

Flow cytometry

A BD FACS ARIA II Flow cytometer was used for both analyzing and positively selecting GFP expression of the tetherin shRNA-transfected cells. Cells were harvested after 2–3 passages and were re-suspended in chilled incomplete medium to prevent the division of cells during the assay.

This was repeated several times to collect the maximum number of GFP-expressing transfected cells. Positively sorted tetherin knockdown cells were cultured as above.

Statistical analyses

All experimental samples were prepared in triplicate in at least 3 separate experiments. Sample *t*-values were calculated using Satterwaite's method for independent samples of unequal variances, and hypothesis testing was employed to determine whether or not quantities were equal, yielding *p*-values indicative of these tests. All error bars represent 95% confidence intervals of a particular dataset, unless otherwise stated.

Results

Tetherin is expressed in murine neuroblastoma cells and is upregulated by IFN-γ and IFN-β

To study the expression of tetherin mRNA in murine neuroblastoma cells (NB41A3), RT-PCR was performed; this revealed the presence of tetherin and β-actin, a house keeping gene (Figs. 1A and 2A). Several previous studies have demonstrated IFN-β induction of tetherin in many different cell types (Ohtomo *et al.*, 1999; Blasius *et al.*, 2006; Neil *et al.*, 2007; Kawai *et al.*, 2008; Miyagi *et al.*, 2009; Weidner *et al.*, 2010; Dietrich *et al.*, 2011). To determine whether type I (IFN-β) or type II (IFN-γ) regulate tetherin mRNA expression in murine neuroblastoma cells, we treated the cells overnight with IFN-β (400 U/mL) or for 72 h with IFN-γ (20 ng/mL). Figure 1A indicates that the expression of tetherin mRNA was upregulated in IFN-treated neurons. There was approximately a 2.5-fold increase in expression of tetherin mRNA in IFN-β-treated neuroblastoma cells (lanes 4–6) compared to the medium-treated neuroblastoma cells and 3-fold by type II IFN (lanes 7–9).

To investigate if tetherin protein levels were induced by IFN-γ or IFN-β treatment, we performed western blot analysis on cell lysates. These data revealed a doubling of tetherin after IFN-β induction as compared to the medium-treated cells (left set of blots and bar graphs). There was more

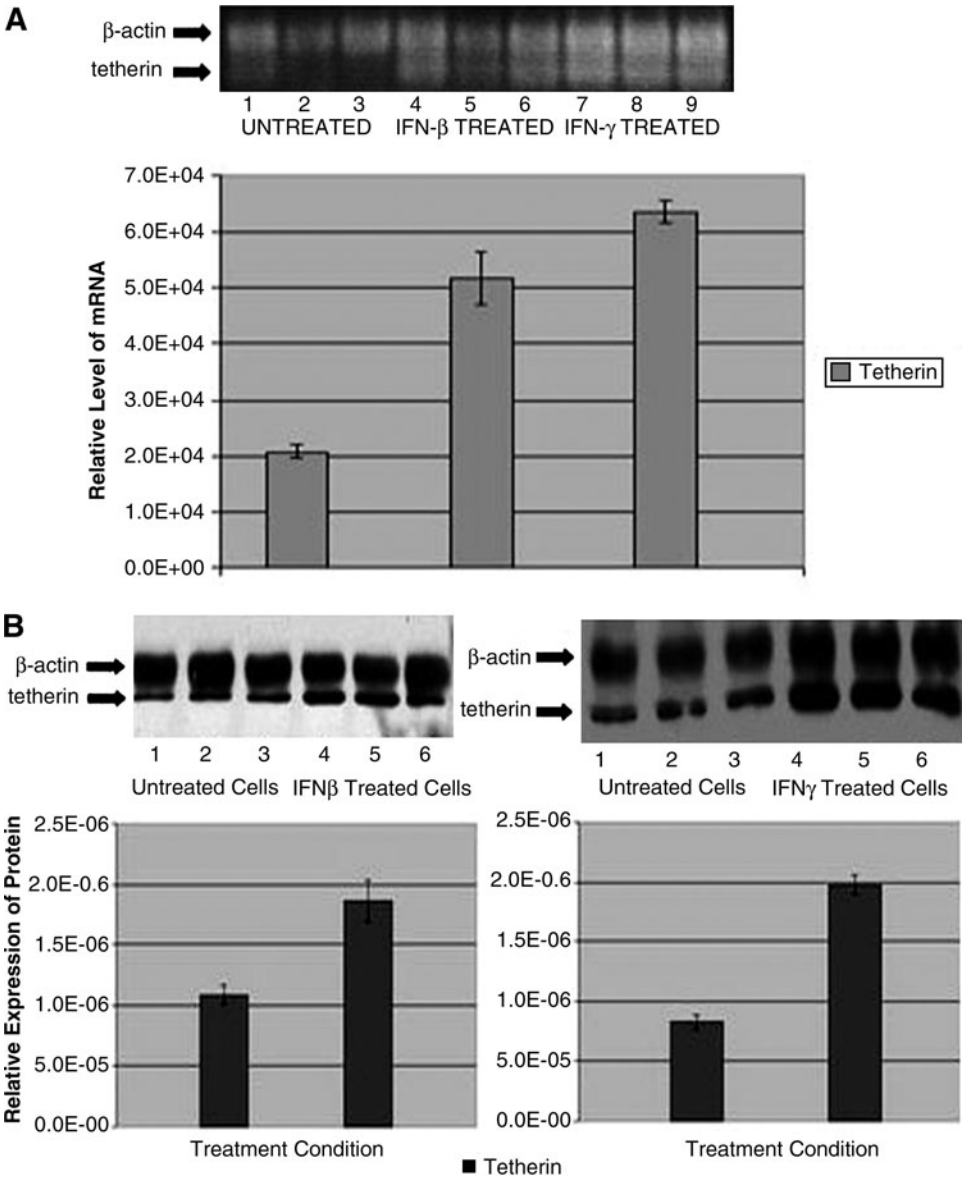

FIG. 1. Tetherin is expressed in neuroblastoma cells and is upregulated by both interferon (IFN)-β and IFN-γ. **(A)** Tetherin mRNA was measured in lysates from control, IFN-β−, or IFN-γ-treated neuronal cells. Replicate cultures were treated with the medium (Control, lanes 1–3), IFN-β (400 U/mL for 24 h, lanes 4–6), or IFN-γ (20 ng/mL for 72 h, lanes 7–9). Tetherin (100 bp) and β-actin (200 bp) bands were detected, and intensities normalized. **(B)** Western blot analyses were performed on cell lysates isolated from medium-treated (lanes 1–3 on both left and right blots and bar graphs), IFN-β-treated (lanes 4–6, left panels), and IFN-γ-treated (lanes 4–6, right panels) samples. Tetherin (28 kDa) and β-actin (36 kDa) were detected. Data of the intensities of the tetherin bands were normalized to β-actin. All experiments were done in triplicates and replicated at least three times.

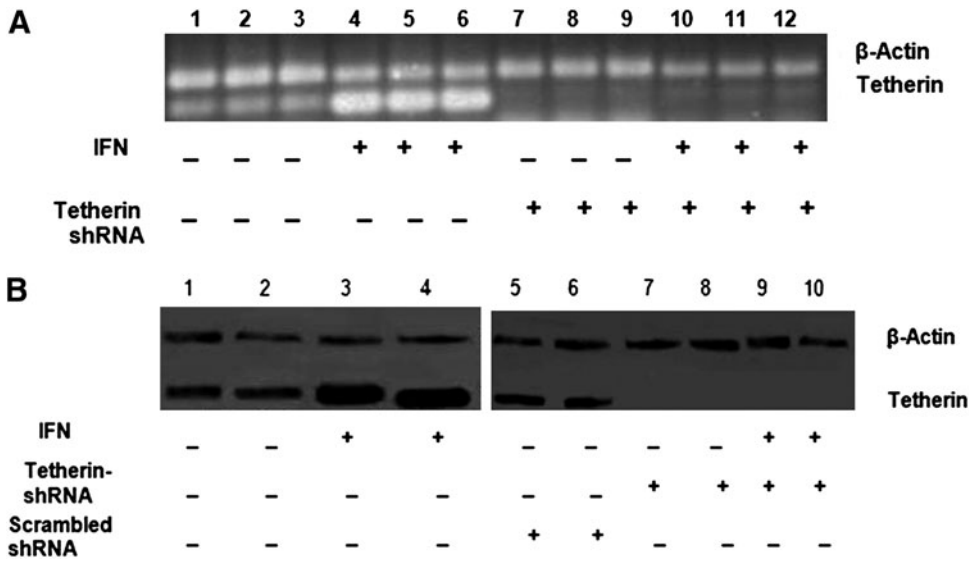

FIG. 2. IFN-β upregulates tetherin expression in neuroblastoma cells, but has no impact on tetherin shRNA-treated cultures. **(A)** Tetherin mRNA expression (100 bp) was detected after either medium or IFN-β treatment, of control neuroblastoma cells and tetherin shRNA cultures. Cell lysates were prepared from medium-treated control neuronal cells (lanes 1–3), IFN-β-treated control cells (lanes 4–6), medium-treated tetherin shRNA cells (lanes 7–9), and IFN-β-treated tetherin shRNA cells (lanes 9–12). β-Actin was used as a housekeeping gene (200 bp). All experiments were performed in triplicate and replicated at least three times. **(B)** Western blot analyses were conducted to study tetherin expression. Cell lysates were prepared from medium-treated control neuronal cells (lanes 1–2), IFN-β-treated control cells (lanes 3 and 4), medium-treated scrambled shRNA-treated neuronal cells (lanes 5 and 6) medium-treated tetherin shRNA cells (lanes 7 and 8) and IFN-β-treated tetherin shRNA cells (lanes 9 and 10). Tetherin was observed at 28 kDa and β-actin at 36 kDa. All experiments were performed in triplicate and replicated at least three times.

than a 2-fold increase in protein expression of tetherin in neuroblastoma IFN-γ-treated neuronal cells as compared to the medium-treated cells (right set of blots and graphs, Fig. 1B).

Silencing the expression of tetherin in neuroblastoma cells using shRNA vectors for infection studies with VSV

We silenced tetherin expression in neuroblastoma cells using synthetic shRNA constructs containing the *tGFP* gene, the expression of which was monitored continuously in the progeny cells to check the efficiency and success of transfection. Two different shRNA cassettes were used in this study: a purified and sequence verified expression plasmid with tetherin-specific shRNA and a purified and sequence verified plasmid containing noneffective 29-mer scrambled shRNA cassette. Transfectants expressing GFP were sorted (Supplementary Figs. S1 and S2, tetherin shRNA and scrambled shRNA, respectively; Supplementary Data are available online at www.liebertonline.com/dna) and expanded. RNA and protein from the transfected cells were isolated and subjected to RT-PCR (Fig. 2A) and western blot analysis (Fig. 2B). The expression of tetherin was completely silenced in tetherin-shRNA-transfected cells, whereas the scrambled shRNA-transfected cells expressed normal levels of tetherin; scrambled shRNA-treated cells were used as controls in further experiments. IFN-β treatment of silenced cells did not induce tetherin mRNA or protein expression.

Morphologically, the tetherin-knockdown cells were smaller and rounder than the control lines. A confocal microscopic analysis also revealed the presence of tetherin in punctate clusters on the outside of the plasma membrane of control neuroblastoma cells (Fig. 3). The tetherin-silenced cells did not exhibit surface tetherin expression, but were positive for expression of a control surface GP, the NMDA receptor. These experiments indicate that we have established tetherin-deficient neuronal cells.

Upregulation of tetherin restricts VSV release in neuroblastoma cells but tetherin knockdown cells are IFN unresponsive for suppressing infectious VSV progeny

After silencing the expression of tetherin in neuroblastoma cells, we examined the replication restriction of VSV virion release in IFN-treated control cells as compared to tetherin-silenced cells. Supernatants were collected at 4, 6, 8, 10, and 12 h postinfection (hpi), and assayed for viral titer on L929 monolayers (Fig. 4A). There was a steady increase in viral titers in supernatants up to 10 hpi in medium-treated neuroblastoma cells (solid bars). In IFN-β-treated control cells, the yield was 10,000-fold less than pfu with medium-treated cells (empty bars), recapitulating our published data (Trottier *et al.*, 2005; D'Agostino and Reiss, 2010).

We performed the VSV infection in medium-treated tetherin-silenced NB41A3 cells and observed that there is 100-fold increase in infectious virus released into the supernatants when tetherin is silenced in neuroblastomas as compared to the normal tetherin-expressing cells (Fig. 4A, slashed bar fill) and there was ~10-fold IFN-mediated inhibition in the tetherin-silenced cells (cross-hatched bar fill). This is consistent with a central role for tetherin in the IFN-mediated antiviral effect in neuronal cells.

Morphological examination of the cells after VSV infection showed a profound difference between IFN-β-pretreated and medium-treated control cells. The medium-treated neuroblastoma cells showed clumping of the cells with a slightly rounded morphology, a cytopathic effect, reflecting the development of apoptosis, as early as 4 hpi and continuing until 12 hpi with dead cells floating in the medium. In contrast, the IFN-β-pretreated control neuronal cells exhibited a

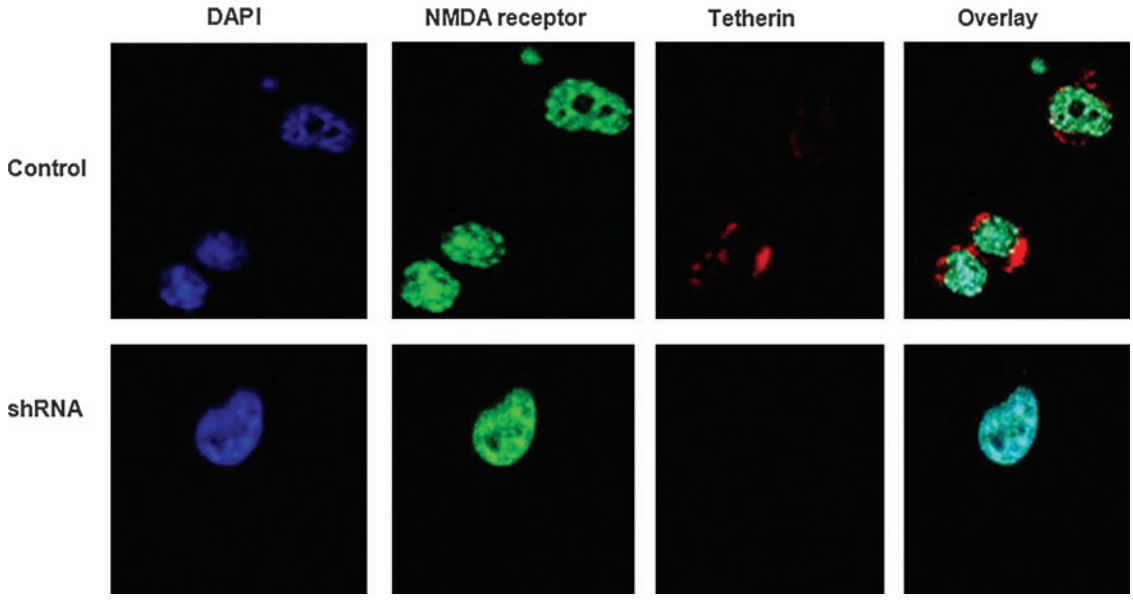

FIG. 3. Detection of tetherin on the outer surface of neuronal cells but not on the silenced neuronal cells. Control NB41A3 (top row) and the tetherin-silenced neuronal cell line (bottom row) were incubated with anti-tetherin (red secondary antibody) and anti-NMDA-R1 (green secondary antibody) antibodies and afterward fixed and permeabilized and nuclei were detected with DAPI stain (blue). Slides were examined using a Leica confocal microscope. Images were overlaid. Images shown are representative of many fields from three replicate experiments. Color images available online at www.liebertonline.com/dna

spread out morphology with few dead cells, even at 12 hpi. Tetherin-silenced cells were more clumped and exhibited a rounded appearance both in uninfected and VSV-infected conditions. The number of floating cells was higher in the infected-silenced cells, and there was no difference in the appearance of IFN-treated, VSV-infected knockdown cells (data not shown).

We had previously observed an inhibition, but not ablation, of viral protein expression in IFN-treated neuronal cells (Trottier *et al.*, 2005; D'Agostino *et al.*, 2009a; D'Agostino and Reiss, 2010). Western blot analysis for expression of viral proteins (G, N and P, and M) was performed after extracting the cell lysates 8–9 hpi from neuroblastoma cells and tetherin-silenced cells (both IFN-β-treated and medium-treated). The results showed the presence of VSV proteins in tetherin-silenced cells (both medium-treated and IFN-β treated), and tetherin-expressing neuroblastoma cells (both medium-treated and IFN-β treated). GAPDH was used as loading control. The intensity of VSV protein bands was comparatively higher in tetherin-silenced cells than tetherin-expressing neuroblastoma cells and much greater in mock-treated than in IFN-treated cells, whether control or tetherin-silenced (Fig. 4B). Thus, IFN treatment was effective in inhibiting some of the viral protein synthesis, but not in preventing virus release from tetherin-silenced cells.

Discussion

Murine neuroblastoma cells used in this research are originally derived from neoplastic tumors and also they are similar in responses to IFN treatment of primary olfactory neurons (Schubert *et al.*, 1969; Breakfield *et al.*, 1975; Chesler *et al.*, 2003). In this research we demonstrated that tetherin is expressed in neuronal cells and is upregulated by both IFN-γ and IFN-β (Fig. 1). The mRNA and protein studies demonstrated upregulation of tetherin by IFN-β approximately 2.5 and 2-fold, respectively. After IFN-γ treatment, a 3-fold increase in mRNA and greater than 2-fold increase in protein were observed. Induction of tetherin by IFNs in non-neuronal cells has been studied by many authors in different cell types and concluded to be cell type-dependant (Neil *et al.*, 2007; Van Damme *et al.*, 2008; Miyagi *et al.*, 2009; Rong *et al.*, 2009).

We used shRNA expressed on a plasmid to transfect the neuronal cells and develop tetherin knocked down lines. They were positively selected by puromycin and flow cytometry (Supplementary Fig. S1) and expanded before characterization. RNA and protein analysis (Fig. 2A, B) confirmed the successful knockdown in the expression of tetherin, with mRNA and protein undetectable in cell lysates, and on the neuronal cell surface (Fig. 3).

Published immunofluorescence studies using 293T cells indicated punctate cell surface expression of tetherin (Hinz *et al.*, 2010), we saw the same distribution of tetherin in our confocal microscopic analysis of neuroblastoma cells (Fig. 3). Confocal microscopic images show the presence of NMDA receptor uniformly expressed on control and shRNA-transfected cells, but not tetherin, which was silenced (Fig. 3). Expression of tetherin was below the level of detection on transfected, GFP-expressing cells, even after treatment with IFN-β, consistent with gene silencing (not shown). Morphological examination of tetherin-silenced cells in our study indicated smaller and rounder cells as compared to the neuroblastoma cells. An explanation for the altered cell appearance may be the interaction of tetherin with the cytoskeleton (Rollason *et al.*, 2009).

In humans, chromosome number 19p13.2 carries a single copy of the tetherin gene, which is organized as four exons

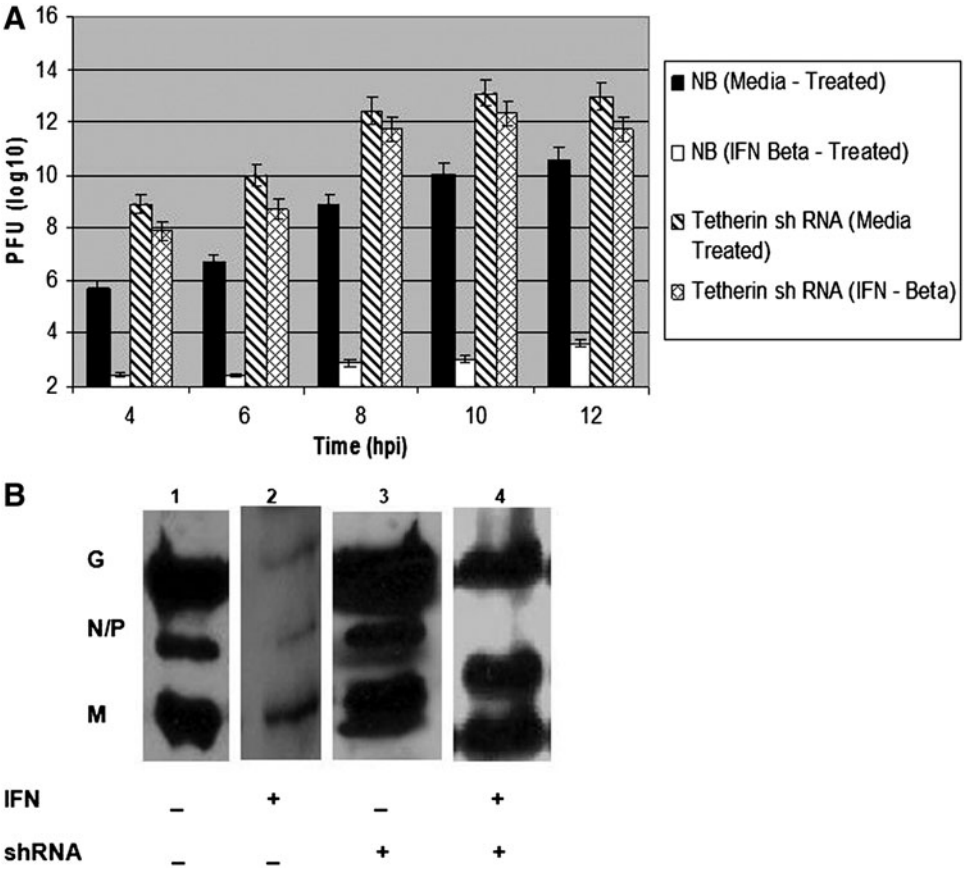

FIG. 4. Impact of tetherin expression on vesicular stomatitis virus (VSV) replication. **(A)** Supernatants from VSV-infected neuronal cells were assayed for infectious virus by plaque assay. Control neuronal cells or tetherin-silenced cells were incubated with the medium (solid fill or slashed fill, respectively) or with IFN-β (empty bars or cross-hatched bar fill, respectively) for 24 h before infection at 3 multiplicity of infection (moi). Supernatants from triplicate cultures were collected at 4, 6, 8, 10, and 12 h postinfection (hpi) and serially diluted before adding to L929 cells. The bars represent mean plaque forming units detected. Error bars represent 3 SD; the experiment was replicated three times. **(B)** Expression of VSV G, N, P, and M proteins was determined by western blot analyses performed with cell lysates (8–9 hpi) of neuroblastoma cells (lanes 1 and 2) and tetherin-silenced cells (lanes 3 and 4) pretreated with the medium (lanes 1 and 3) or with IFN-β-treated (lanes 2 and 4) for 24 h. The bands were 55 kDa for VSV G protein, 45 kDa for VSV N and P proteins, and 25 kDa for VSV M protein. Mouse GAPDH was used as loading control. Data are representative of three replicate experiments, each with triplicate cultures at each condition.

and is nonpolymorphic (Sugiyama *et al.*, 1995). Orthologs of this occurs in Old World monkeys and placental mammals. Several cell types, including T cells, B cells, monocytes, macrophages, and plasmacytoid dendritic cells (PDC), express tetherin constitutively (Sugiyama *et al.*, 1995; Vidal-Laliena *et al.*, 2005). Before this report, tetherin expression has not been described on neurons.

The virions retained by tetherin undergo endocytosis followed by lysosomal degradation (Neil *et al.*, 2008; Hammonds and Spearman, 2009; Sato *et al.*, 2009). The destruction of virions has been shown by biochemical and immunoelectron microscopic methods (Evans *et al.*, 2010). Tetherin forms a bridge between virions and the plasma membrane (Sato *et al.*, 2009). Tetherin contributes to antiviral activity against various enveloped viruses, including Ebola, Marburg, Lassa, KSHV, HIV-1, HIV-2, SIV, MLV, E1AV, VSV, HTLV-1, XMRV, and spumaviruses (Jouvenet *et al.*, 2009; Kaletsky *et al.*, 2009; Le Tortorec and Neil, 2009; Mansouri *et al.*, 2009; Miyakawa *et al.*, 2009; Sakuma *et al.*, 2009; Groom *et al.*, 2010; Weidner *et al.*, 2010; Xu *et al.*, 2011).

Many viruses have evolved pathways to evade host antiviral mechanisms by disabling the IFN pathway (Versteeg and Garcia-Sastre, 2010) or by using proteins to antagonize tetherin activity and remove it from the plasma membrane. The coimmunoprecipitation of HIV-1 vpu and tetherin results from the interaction of their transmembrane domains, and this association was found to be critical for reducing cell-surface tetherin expression (Dube *et al.*, 2010; Jolly *et al.*, 2010). Tetherin and HIV-1 Gag accumulate at the contact zone between infected and target cells, but tetherin cannot prevent the formation of viral synapses; together, tetherin and HIV-1 virions are transferred to target cells as abnormally large patches that are impaired in their fusion capacities (Casartelli *et al.*, 2010).

Studies with the Ebola virus GP show evasion the antiviral effect of both murine and human tetherin and coimmunoprecipitation. Studies indicate that Ebola GP is active against an artificial tetherin construct without removing the protein from the cell surface, suggesting that it is possible to overcome this restriction by mechanisms other than blocking tetherin's cell surface expression (Lopez *et al.*, 2010).

Similarly, the K5 protein of KSHV (HHV8) antagonizes conserved lysine residues of primate tetherin by targeting it for ubiquitination (Pardieu *et al.*, 2010). K5 induces a species-specific downregulation of human tetherin and endosomal degradation as well. SIV negative regulatory factor (Nef) antagonizes nonhuman primate orthologs of tetherin (Bartee *et al.*, 2006; Gupta *et al.*, 2009; Jia *et al.*, 2009; Zhang *et al.*, 2009).

Cotranslational movement of tetherin into endoplasmic reticulum, transportation through the Golgi apparatus in COPII-coated vesicles to the plasma membrane comprises its

expression pattern (Rollason *et al.*, 2007). In PDCs, tetherin inhibits the expression of type 1 IFNs and other proinflammatory cytokines to prevent hyper activation of immune system by binding to immunoglobulin-like transcript 7 (ILT7) (Cao *et al.*, 2009). Experiments using artificial tetherin suggest that it may be possible to develop tetherin-based restriction factors to prevent a wide range of viral infections and which could also overcome the evasion of antagonists produced by many viruses (Perez-Caballero *et al.*, 2009).

As we have previously demonstrated, neuroblastoma cells pretreated with IFN-β showed a considerable decrease in both viral proteins in lysates and viral yields from supernatants (Trottier *et al.*, 2005; D'Agostino *et al.*, 2009a) than medium-treated cells, consistent with the powerful antiviral cell-autonomous response induced by IFN-β. There was approximately a 10,000-fold decrease in viral titers obtained from IFN-β-treated samples as compared to medium-treated cells (Fig. 4A). After knocking down the expression of tetherin, medium-treated neuronal cells were found to yield more than 100-fold more virus after VSV infection. Our observation was consistent with the results from Ju-Tao Guo's laboratory obtained from a BHK-21 FLP-IN T Rex-derived stable tetherin knockdown cell line (Weidner *et al.*, 2010); they did not assess the role of IFN treatment on the tetherin-deficient line. We found that there was no significant difference between the viral yield in medium-treated tetherin shRNA cells and IFN-β-treated tetherin-silenced neuronal cells (Fig. 4A). This unexpected finding is consistent with a primary role of tetherin in the antiviral effect of IFN in neuronal cells.

Previously, we had observed that IFN treatment has a significant effect on the levels of VSV viral proteins produced in neuronal cells, but that PKR was not induced (Trottier *et al.*, 2005); we assessed the impact of IFN treatment of the knockdown cells by western blot analyses of cell lysates at 8–9 hpi. VSV proteins G, N, P, and M were expressed more highly in neuroblastoma (medium-treated) cells, than the IFN-β pretreated control neuroblastoma cell lysates. The expression of VSV proteins were substantially higher in tetherin knocked down cells than control neuronal cells, but IFN treatment did (modestly) inhibit VSV protein synthesis in the tetherin shRNA cells (Fig. 4B).

Clearly, additional cell autonomous antiviral pathways, including altered kinase/phosphatase activity, are regulated by IFN-γ and impede viral protein expression (D'Agostino *et al.*, 2009b). Thus, IFN treatment reduced viral protein production in the tetherin-silenced cells, but was unable to suppress release of assembled infectious progeny virions.

Conclusions

We have demonstrated that tetherin is constitutively expressed by murine neuronal cells. Data obtained from this research clearly indicate the importance of tetherin in contributing to the cell autonomous antiviral immune response in mammalian neuronal cells by restricting the release of virions. Tetherin expression in neurons is positively regulated by both type I and type II IFN. Tetherin is expressed in a punctate pattern on the surface of neurons. Tetherin-silenced cells were functionally unresponsive to the antiviral actions of IFN treatment.

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Disclosure Statement

No competing financial interests exist.

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