Short Communication

Identification of *Japanese encephalitis virus*-inducible genes in mouse brain and characterization of GARG39/IFIT2 as a microtubule-associated protein

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Several mouse central nervous system genes have been identified that are differentially regulated during *Japanese encephalitis virus* (JEV) infection, including those which have not been reported to be induced by any other neurotropic virus. Interestingly, ~80 % of JEV-inducible genes identified in this study are also induced by *Sindbis virus*, indicating activation of common host signalling pathways by these two viruses, despite their diverse life cycles. One of these, the glucocorticoid attenuated response gene 39 (GARG39, also known as IFIT2, ISG54 and MuP54) was characterized further. It was demonstrated that GARG39 protein interacts with microtubules *in vitro*, co-localizes with β-tubulin *in vivo* and is enriched in the mitotic spindle of non-neuronal cells undergoing mitosis. While GARG39 was known for a long time as an inflammation-inducible glucocorticoid attenuated protein, its identification as a microtubule-associated protein in this study suggests a possible role for this protein in cell proliferation, virion assembly/transport and microtubule dynamics.

One of the key events in a virus-infected host cell is the activation or repression of a large number of host genes. In a previous study, employing subtraction hybridization strategy, we identified nine mouse central nervous system (CNS) genes which are up-regulated during *Japanese encephalitis virus* (JEV) as well as *Rabies virus* (RABV) infection (Saha & Rangarajan, 2003). Further screening of a subtracted cDNA library led to the identification of an additional 24 JEV-inducible genes whose up-regulation in JEV-infected mouse brain was confirmed by Northern blotting analysis (Fig. 1). The expression of many of these genes peaks at day 6 post-infection (Fig. 1, lane 3) when the virus load is maximum in the mouse brain following intracerebral inoculation of JEV (Saha & Rangarajan, 2003). In all these studies, 6- to 8-week-old outbred Swiss mice were inoculated intracerebrally with a 10 LD_{50} dose of the mouse-adapted neurovirulent JEV strain P20778, which results in 100 % mortality within 8–10 days (Saha & Rangarajan, 2003).

The JEV-inducible mouse CNS genes identified in this study were classified into two categories. The first category includes LRG-21, VHSV-induced gene 1, Tpt1, SLC25A3, Olfm1, Ina/NF-66, Dst/Bpag1, Mdm2, Gbp5 and an unannotated gene bearing GenBank accession no. AK028745 which are not reported to be induced by any other neurotropic virus (Booth *et al.*, 2004; Gruslin *et al.*, 2005; Johnston *et al.*, 2001; Prosniak *et al.*, 2001; Saha & Rangarajan, 2003; Labrada *et al.*, 2002; Koh & Ng, 2005; Venter *et al.*, 2005). Thus, these genes are either unique to JEV or have been overlooked by others in their screening due to differences in the technologies used and in the sensitivity of detection of differentially expressed genes. While some of these genes still remain to be annotated, the function of many others is still not well understood, especially in the CNS. For example, LRG-21 encodes a transcription factor with homology to c-Fos/c-Jun and is induced by lipopolysaccharides, interferon-γ and interleukin-4 in macrophages (Drysdale *et al.*, 1996). However, its exact function is not known. TPT1 (also known as TCTP) is a calcium- and microtubule-binding protein implicated in important cellular processes, such as cell growth, cell-cycle progression, malignant transformation and in the protection of cells against various stress conditions and apoptosis (Bommer & Thiele, 2004). Rotaviral infection or expression of NSP4, a rotavirus non-structural glycoprotein is known to increase TPT1 mRNA levels by transcriptional as well as post-transcriptional regulatory mechanisms. TPT1 is known to function as a molecular chaperone in the lumen of the endoplasmic reticulum (ER), suggesting a possible role in the synthesis, assembly and maturation of viral proteins in

A table showing the expression profile of JEV-inducible mouse CNS genes in other neurotropic viral infections is available as supplementary material in JGV Online.

1	2	3	Gene name	Genbank no.
			Interferon-inducible protein 1 (LRG-47/Ifi1)	NM_008326
			Interferon-inducible GTPase (IIGP1)	BC004649.1
			Interferon-γ-induced GTPase (IGTP)	NM_018738.1
			T-cell-specific GTPase (TGTP/Mg21)	NM_011579.2
			Interferon regulatory factor 7 (IRF7)	NM_016850
			Interferon-activated gene 202B (Ifi202b)	BC018233.1
			Chemokine (C-X-C) motif ligand 10 (Cxcl10)	BC030067.1
			Histocompatibility 2, D region locus 1 (H2-D1)	XM_110204.1
			Granzyme B	BC002085.1
			Lysophospholipase (Lyp1a1)	NM_013006.1
			Cyclophilin c-associated protein (CyC-AP)	NM011150.1
			Fc fragment of IgG, low-affinity IIIa (Fcgr3a)	NM_144559.1
			Glucocorticoid attenuated response gene 16 (GARG16)	U43084.1
			Glucocorticoid attenuated response gene 39 (GARG39)	U43085.1
			LRG21	MMU19118
			Viral hemorrhagic septicemia virus (VHSV-induced gene 1)	AK010906.1
			Tumour protein, translationally controlled (Tpt1)	XM_124760.1
			Solute carrier family 25, member 3 (Slc25a3)	BC018161.1
			Olfactomedin 1 (Olfm1)	AK011705.1
			Internexin neuronal intermediate filament protein (Ina/NF-66)	NM_146100
			Dystonin (Dst/Bpag1)	NM_133833.1
			Mus musculus double minute (Mdm2)	AF320762.1
			Guanylate-binding protein 5 (Gbp5)	XM_149395.1
			Virus-inducible non-coding RNA (VINC)	AK028745
			GAPDH	

Fig. 1. Identification of JEV-inducible genes in mouse brain. Northern blots containing brain RNA isolated from JEV-infected mice at days 3 (lane 2) and 6 (lanes 3) post-intracerebral inoculation were probed with various radiolabelled mouse cDNAs isolated by subtraction hybridization, as described by Saha & Rangarajan (2003). Brain RNA isolated from mice injected with saline and sacrificed 6 days later (lane 1) served as the control. The blots were rehybridized with a probe for GAPDH to monitor RNA loading.

the ER (Xu *et al.*, 1999). SLC25A3 is involved in phosphate transport into the mitochondrial matrix and is expressed in a number of non-neuronal tissues (Palmieri, 2004). However, its expression in the CNS has not been reported thus far. Olfm1 is a neuron-specific mRNA encoding a protein with high homology to olfactomedins which are implicated in the maintenance, growth or differentiation of chemosensory cilia on the apical dendrites of olfactory neurons (Ando *et al.*, 2005; Danielson *et al.*, 1994). Ina/NF-66 encodes α-internexin whose overexpression results in several motor neuron diseases, including neuronal intermediate filament inclusion disease (Armstrong *et al.*, 2005). Interaction of α-internexin with human T-cell leukaemia virus (HTLV) transcriptional transactivator protein, Tax, has also been reported (Reddy *et al.*, 1998). Bpag1/dystonin proteins are cytoskeletal interacting proteins whose loss of function results in neuromuscular dysfunction and early postnatal death in mice (Young *et al.*, 2006). GBP-5 is a new member of the interferon (IFN)-inducible guanylate-binding protein (GBP) family and has not yet been fully characterized (Nguyen *et al.*, 2002). The unannotated gene bearing GenBank accession no. AK028745 has recently been shown to encode a virus-inducible non-coding RNA (Saha *et al.*, 2006).

The second category of JEV-inducible genes include those which are already reported to be activated in mouse CNS by other neurotropic viruses such as RABV (Prosniak *et al.*, 2001), *Sindbis virus* (SINV) (Johnston *et al.*, 2001; Labrada *et al.*, 2002), scrapie agent (Booth *et al.*, 2004), coronavirus (Gruslin *et al.*, 2005) and *West Nile virus* (WNV) (Koh & Ng, 2005; Venter *et al.*, 2005). These genes are listed in Table S1 (available as supplementary material in JGV online). Many of these genes have established roles in the host response to viral infection, such as interferon signalling, antigen presentation and processing, chemokine signalling, lymphocyte proliferation and the cytotoxic T lymphocyte response (Booth *et al.*, 2004; Gruslin *et al.*, 2005; Johnston *et al.*, 2001; Prosniak *et al.*, 2001; Saha & Rangarajan, 2003). Interestingly, 19 out of 24 genes listed in Table S1 (available as supplementary material in JGV online) are common for JEV and SINV. These include genes encoding IFN-inducible GTPases (LRG-47, IIGP1, IGTP, TGTP), transcription factors involved in the regulation of interferon genes (IRF-7, STAT1), the lysosomal cysteine protease cathepsin S, 2′-5′ oligoadenylate synthetase (OAS) involved in murine flavivirus resistance, IFN-activated gene 202A and glucocorticoid-attenuated response genes (GARG16, GARG39,

GARG49) (Johnston *et al.*, 2001; Labrada *et al.*, 2002; Saha & Rangarajan, 2003). These results indicate that, despite diverse life cycles, these two viruses activate common host signalling pathways.

Among the common genes activated by JEV and other neurotropic viruses, we focused our attention on the glucocorticoid attenuated response genes, especially GARG39/IFIT2, which is induced in mouse brain by not only JEV, but also SINV (Johnston *et al.*, 2001; Labrada *et al.*, 2002) and WNV (Koh & Ng, 2005; Venter *et al.*, 2005). GARG39, together with GARG49 and GARG16, were first identified as lipopolysaccharide (LPS)-induced, dexametha-sone-attenuated transcripts in Swiss 3T3 fibroblasts (Smith & Herschman, 1995). They belong to a highly conserved family of proteins containing multiple tetratricopeptide repeat (TPR) domains (Smith & Herschman, 1996, 2004; Sarkar & Sen, 2004). However, the expression of GARG39 has not been examined at the protein level in any cell type thus far. We therefore isolated the full-length GARG39 cDNA from JEV-infected mouse brain RNA by RT-PCR using the primer pair 5′-CGCCGCGGAT-CCATGAGTACAACGAGTAAGGAG-3′ and 5′-AAACCG-CTCGAGCTAGTATTCAGCACCTGC-3′. The cDNA was cloned into a bacterial expression vector (pRSETA; Invitrogen), the recombinant protein was expressed as a histidine-tagged protein and purified by Ni^{2+}-agarose chromatography (Fig. 2a). Polyclonal antibodies were raised in rabbit against recombinant GARG39 and their specificity was determined by Western blotting analysis of mouse brain homogenates. Anti-GARG39 antibodies reacted only with a ~55 kDa protein corresponding to the molecular mass of GARG39 in JEV-infected (Fig. 2b, lane 2) but not normal (Fig. 2b, lane 1) mouse brain homogenate. Since GARG39 mRNA is induced in many cell lines by LPS and interferons (Smith & Herschman, 1996, 2004; Sarkar & Sen, 2004), subcellular localization studies of GARG39 protein were carried out in mouse NIH3T3 cells.
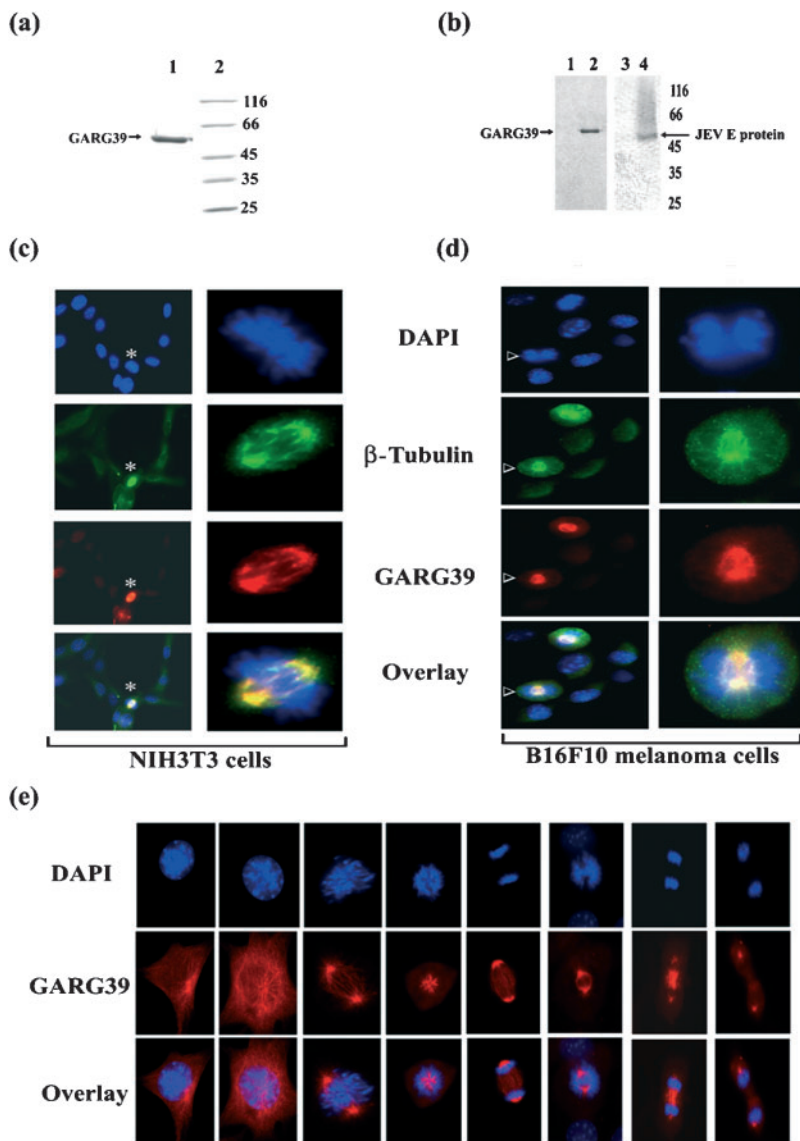

Fig. 2. Subcellular localization of GARG39 protein. (a) SDS-PAGE analysis of purified recombinant GARG39 (lane 1). Numbers indicate protein molecular mass (kDa) markers (lane 2). (b) Western blot analysis of brain homogenates isolated from normal (lanes 1, 3) or JEV-infected mice (lanes 2, 4). Blots were probed with anti-GARG39 antibodies (lanes 1, 2) or anti-JEV envelope (E) protein antibodies. (c, d) Immunolocalization of GARG39 in normal NIH3T3 and B16F10 melanoma cells. GARG39 was detected using anti-GARG39 antibodies and rhodamine-conjugated goat anti-rabbit IgG. β-Tubulin was detected using mouse anti-β-tubulin antibodies (Santa Cruz) and FITC-conjugated goat anti-mouse IgG. Nuclei were stained by 4′, 6-diamidino-2-phenylin-dole (DAPI). The right panels show an enlarged image of mitotically active cells marked in the left panels by an asterisk (c) or triangle (d). (e) Association of GARG39 with mitotic spindle in NIH3T3 cells at various stages of mitosis.

Interestingly, GARG39 could be readily visualized by immunofluorescence in the mitotic spindle of a small fraction of mitotically active normal, uninfected NIH3T3 cells (Fig. 2c). Similar results were obtained with B16F10 mouse melanoma cells as well (Fig. 2d). β-Tubulin, a key component of the mitotic spindle was used as a marker in these studies. GARG39 could be visualized in the mitotic spindle during various stages of mitosis in NIH3T3 cells (Fig. 2e). To examine whether GARG39 interacts with tubulin *in vitro*, co-sedimentation assays were carried out using purified recombinant GARG39 and taxol-stabilized tubulin polymers isolated from mouse brain essentially as described by Ding *et al.* (2006) and Hoffner *et al.* (2002). Recombinant GARG39 could be co-sedimented more efficiently in the presence of tubulin polymer than in its absence (Fig. 3), indicating that GARG39 is a microtubule-associated protein.

GARG39 mRNA was shown to be induced by interferon-α/β in a STAT1-dependent manner in primary neuronal cultures (Wang & Campbell, 2005). Comparison of gene expression changes in the CNS induced by different neurotropic viruses indicated a good correlation between virus-induced activation of STAT1 and GARG39 in the mouse CNS, as was evident from their co-activation by JEV, SINV and WNV (Table S1, available as supplementary material in JGV online). Results from our laboratory indicate that GARG39 mRNA is induced in mouse brain by RABV as well (data not shown). Whether JEV-induced activation of GARG39 in mouse CNS also occurs primarily in neurons is not clear from the present study. The association of GARG39 with microtubules and its co-localization with β-tubulin in the mitotic spindle indicates a possible role for this protein in mitosis. Thus, GARG39 is readily detectable in uninfected NIH3T3 as well as B16F10 melanoma cells because of their high mitotic activity. In the normal mouse brain, mitotically active cells are very low in

number and hence GARG39 expression is not detectable at either the RNA and protein levels. JEV infection may trigger mitotic activity in the brain, leading to increased expression of GARG39. In addition, a possible role for GARG39 in virus survival cannot be ruled out since microtubules and microtubule-associated proteins are known to play an important role in the intracellular trafficking of viral components as well as transportation of virions in the infected host cell (Greber & Way, 2006; Radtke *et al.*, 2006). In fact, an interaction between TSG101, a microtubule-associated protein, and JEV NS3 protein has been documented (Chiou *et al.*, 2003). Whether GARG39 interacts with any of the viral proteins through its TPR domains remains to be examined. Since microtubule-associated proteins are implicated in several neurodegenerative disorders, including Parkinson's disease, Alzheimer's disease and mental retardation (Brown *et al.*, 2001; Dickson, 1997; Lewis *et al.*, 2000), a role for GARG39 in virus-induced neuropathogenesis cannot be ruled out. In addition to its up-regulation in the brain during virus infection, its abundant expression in mitotically active non-neuronal cells in culture and its association with the mitotic spindle indicate a role for GARG39 during cell division. Although GARG39 mRNA was identified as an LPS- and interferon-inducible transcript more than a decade ago (Smith & Herschman, 1995), the subcellular localization and function of the GARG39 protein is not known. Thus, the identification of GARG39 as a microtubule-associated protein in this study may pave way for the study of its role in cell proliferation, virus infection, virus-induced neuropathogenesis, antiviral response and microtubule dynamics.
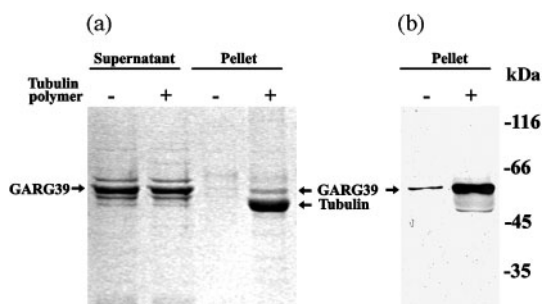
Acknowledgements

Fig. 3. *In vitro* tubulin pull-down assay. Taxol-stabilized microtubules were prepared from mice brain (Hoffner *et al.*, 2002), incubated with recombinant GARG39 protein and centrifuged at 100 000 *g*. The supernatant and pellet fractions were analysed by SDS-PAGE and tubulin-dependent co-sedimentation of GARG39 in the pellet fraction was demonstrated by Coomassie blue staining of the gel (a) as well as by Western blot analysis using anti-GARG39 antibodies (b).

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