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Type 1 IFN-independent activation of a subset of interferon stimulated genes in West Nile virus Eg101-infected mouse cells

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ABSTRACT

Although infection of mouse embryofibroblasts (MEFs) with WNV Eg101 induced interferon (IFN) beta production and STAT1 and STAT2 phosphorylation, these transcription factors (TFs) were not detected in the nucleus or on the promoters of four IRF-3-independent interferon stimulated genes (ISGs): Oas1a and Irf7 (previously characterized as IFN/ISGF3-dependent), Oas1b and Irf1. These ISGs were upregulated in WNV Eg101-infected STAT1−/−, STAT2−/−, and IFN alpha/beta receptor−/− MEFs. Although either IRF-3 or IRF-7 could amplify/sustain Oas1a and Oas1b upregulation at later times after infection, these factors were not required for the initial gene activation. The lack of upregulation of these ISGs in WNV Eg101-infected IRF-3/9−/− MEFs suggested the involvement of IRF-9. Activation of Irf1 in infected MEFs did not depend on any of these IRFs. The data indicate that additional alternative activation mechanisms exist for subsets of ISGs when a virus infection has blocked ISG activation by the canonical IFN-mediated pathway.

Introduction

The family *Flaviviridae*, genus *Flavivirus*, contains several human pathogens including West Nile virus (WNV), dengue virus, yellow fever virus (YFV), Japanese encephalitis virus (JEV) and tick borne encephalitis virus (TBEV). WNV is a mosquito-borne virus that is transmitted in a bird–mosquito cycle but occasionally mammals including humans and horses are infected. WNV infections in humans are usually asymptomatic but can induce a mild febrile illness; however, some patients develop encephalitis or a poliomyelitis-like disease. WNV particles contain a single-stranded, positive-sense RNA genome encoding a single polyprotein that is cleaved by viral and cellular proteases into three structural and seven nonstructural proteins (Brinton, 2002). WNV isolates have been divided into two main lineages. Lineage I strains are often associated with outbreaks of human disease, while the majority of lineage II strains are non-emerging and cause zoonotic infections in Africa (Brinton, 2002).

IFNs were first discovered as cytokines that inhibit viral replication (Isaacs and Lindenmann, 1957). Induction of type I IFNs is primarily triggered by pattern recognition receptors (PRRs) that recognize viral nucleic acids. Viral double stranded RNA (dsRNAs) or single stranded RNAs (ssRNAs) are recognized by cytoplasmic PRRs, such as retinoic acid inducible gene-I (RIG-I) and melanoma differentiation antigen 5 (MDA5), or by cell surface and endosomal PRRs, such as Toll-like receptor 3 (TLR-3), TLR7 and TLR-8 (Saito and Gale, 2007). Recognition of viral nucleic acids by these PRRs leads to activation of the TFs, IRF-3, NF-kappa B and ATF2/c-Jun (AP1), that assemble on the IFN-beta promoter and activate IFN-beta gene expression (Merika and Thanos, 2001). Secreted IFN beta binds to cell surface IFN alpha/beta receptor (IFN alpha/beta R) complexes inducing activation of receptor-associated Jak kinases followed by recruitment and phosphorylation of the TFs, STAT1 and STAT2. STAT1, STAT2 and IFN regulatory factor-9 (IRF-9) form a trimeric transcription factor complex referred to as IFN stimulated gene factor 3 (ISGF3) that translocates to the nucleus and binds to IFN-stimulated response elements (ISREs) in the promoters of IFN-stimulated genes (ISGs) (Stark et al., 1998). The induction of IFN beta is biphasic due to the induction of ISGs, such as IRF-7, which enhance IFN beta gene expression and sustain ISG production. IRF-7 also induces the expression of IFN-alpha which amplifies the type I IFN response via a positive feedback loop (Marie et al., 1998; Sato et al., 2000).

The expression of IFN beta and multiple interferon stimulated genes (ISGs), some of which have identified antiviral functions, represents the first line of defense against a viral infection. Many viruses suppress the innate immune response by blocking the Jak-STAT signaling pathway. Different flaviviruses were reported to block this pathway by either inhibiting Jak phosphorylation (Best et al., 2005; Guo et al., 2005; Ho et al., 2005; Lin et al., 2004), reducing the expression of STAT2 (Jones et al., 2005; Mazzon et al., 2009) or blocking STAT1 and STAT2 phosphorylation (Best et al., 2005; Liu et al., 2005;

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Munoz-Jordan et al., 2003, 2005). The lineage I WNV strains TX02 and NY99 were previously shown to effectively block STAT1 phosphorylation in primate cells (Keller et al., 2006; Laurent-Rolle et al., 2010; Liu et al., 2005). In contrast, WNV Eg101, a lineage I stain closely related to NY99 but less neuroinvasive (Beasley et al., 2002) was previously reported not to inhibit STAT1 phosphorylation in mouse cells (Scherbik et al., 2006, 2007). However, the effects of WNV Eg101 infection on STAT2 phosphorylation and ISGF3 nuclear translocation in these cells were not investigated.

Inhibition of the Jak-STAT signaling pathway by a viral infection would be expected to suppress ISG expression which is required for the establishment of an effective cellular antiviral response. However, even though ISGs were first described as IFN-induced genes, some were subsequently shown to be upregulated by viral infections in an IFN-independent manner through recognition of viral components by PRRs and activation of constitutively expressed TFs such as IRF-3 and NF-kappa B (Andersen et al., 2008; Basagoudanavar et al., 2011; Elco et al., 2005; Grandvaux et al., 2002; Nakaya et al., 2001; Peters et al., 2002). Subsequent upregulation of additional TFs, such as IRF-7, was also shown to play a role in the expression of some ISGs in infected cells (Barnes et al., 2004). IRF-7 and IRF-3 were previously reported to be important for IFN production and protection against WNV (Daffis et al., 2007, 2008; Fredericksen et al., 2004).

Due to the high degree of homology between the ISRE and IRF binding element (IRF-E) consensus sequences, the ISREs of some ISGs can be directly induced by either IRF-3 or IRF-7 (Lin et al., 2000; Morin et al., 2002; Schmid et al., 2010). In MEFs, Irf7 gene expression must first be induced and then IRF-7 has to be activated before this TF can directly upregulate a subset of ISGs (Barnes et al., 2004). In contrast, IRF-3 is a constitutively expressed protein that is in the cytoplasm of all cells in an inactive form. IRF-3 activation is mediated by phosphorylation at multiple C terminal serine and threonine residues. Phosphorylation-induced dimerization leads to nuclear translocation of IRF-3 in a complex with histone acetyltransferases p300 and CREB-binding protein (CBP) (Fitzgerald et al., 2003; Sharma et al., 2003; Suhara et al., 2002; Yoneyama et al., 1998). The results of an analysis of murine ISG expression during a Newcastle disease virus (NDV) infection in IRF-3−/− MEFs led to the classification of ISGs such as ISG15, ISG54, IP-10 and GBP as genes activated in an IRF-3-dependent manner and ISGs such as Oas1a and Irf7 as genes upregulated in an IRF-3-independent but IFN-dependent manner through activation of the ISGF3 transcription factor complex (Nakaya et al., 2001). A more recent analysis of ISG expression in IRF-3−/− MEFs infected with NDV confirmed that the expression of Oas1a and Irf7 did not depend on IRF-3 and showed that this was also the case for the Oas1b and Irf1 genes (Andersen et al., 2008).

NF-kappa B exists in the cytoplasm in an inactive form in a complex with an inhibitory I kappa B protein (Whiteside and Israel, 1997). Activation of the NF-kappa B pathway by viral infection upregulates the expression of a specific subset of ISGs encoding cytokines and chemokines, such as RANTES, TNF alpha and others, regulators of apoptosis and TFs, including A20, Irf1 and Irf2 and others (Elco et al., 2005; Pahl, 1999).

The murine 2′–5′ oligoadenylate synthetase 1a (Oas1a) and Oas1b genes are ISGs that have antiviral functions. Oas1a is an enzymatically active synthetase that upon binding to viral dsRNA synthetizes 2′–5′-oligoadenylates (2–5A) from ATP which activate the latent endonuclease RNase L. Activated RNase L degrades both cellular and viral single-stranded RNAs (Samuel, 2001). Oas1b is an inactive synthetase that mediates resistance to flavivirus-induced disease through an unknown RNase L independent mechanism (Scherbik et al., 2006). Oas1b was also reported to inhibit *in vitro* Oas1a synthetase activity in a dose-dependent manner (Elbahesh et al., 2011). Another study showed that IFN beta activation of Oas1a expression was STAT1- and STAT2-dependent while that of Oas1b was STAT1-independent and STAT2-dependent indicating that these two duplicated genes are differentially regulated by IFN beta (Pulit-Penaloza et al., 2012). WNV Eg101 infection in MEFs was previously reported to activate IFN beta expression, induce STAT1 Tyr701 phosphorylation and upregulate the expression of Oas1a, Oas1b as well as other ISGs including Irf7 and Irf1 (Scherbik et al., 2006, 2007). However, whether the upregulation of these IRF-3-independent ISGs in WNV-infected MEF is mediated by IFN or by an alternative virus-activated pathway was not previously analyzed.

Although IFN beta expression is upregulated and STAT1 and STAT2 are phosphorylated in WNV Eg101 infected MEF, the present study showed that nuclear translocation of these TFs was blocked. Consistent with this observation, no increase in the binding of either STAT1 or STAT2 to the Oas1a, Oas1b or Irf7 promoters or of STAT1 to the Irf1 promoter was observed even though these ISGs were upregulated. Each of these genes was also upregulated by WNV Eg101 infection in STAT1−/−, STAT2−/− and IFN alpha/beta R−/− MEFs indicating that these ISGs were not upregulated by the canonical type 1 IFN-mediated Jak-STAT pathway or by an alternative IFN alpha/beta R-mediated signaling pathway. Oas1a, Oas1b and Irf7 were also upregulated in infected IRF-3−/−, IRF-7−/− MEFs and initially in IRF-3/7−/− MEFs but not in infected IRF-3/9−/− MEFs suggesting the involvement of IRF-9. Either IRF-3 or IRF-7 could enhance Oas1a and Oas1b upregulation at later times after infection. Activation of Irf1 in infected MEFs did not depend on any of these IRFs. The data support the existence of alternative mechanisms of ISG upregulation when the canonical type I IFN pathway is blocked by a WNV infection.

Results

The kinetics of IFN beta expression, secretion and signaling in WNV Eg101-infected MEFs

Previous studies reported increased STAT1 phosphorylation as well as increased expression of many ISGs, including Oas1a, Oas1b, Irf1 and Irf7 in WNV Eg101-infected MEFs (Scherbik et al., 2006, 2007). To determine whether ISG expression in WNV Eg101-infected MEFs is temporally related to the induction of IFN beta, the kinetics of IFN beta expression in WNV Eg101 infected [multiplicity of infection (MOI) of 5], transformed C3H/He (tC3H/He) MEFs were analyzed by real time qRT-PCR. IFN beta mRNA levels in tC3H/He MEFs were elevated by 10 fold at 6 h, by 100 fold at 12 h and by more than 5000 fold at 24 and 48 h after WNV Eg101 infection (Fig. 1A). The results obtained were similar to those previously reported for infected primary C3H/He (pC3H/He) MEFs (Scherbik et al., 2006). Analysis of extracellular IFN beta protein levels with an enzyme-linked immunosorbent assay (ELISA) detected low levels of IFN beta at 16 h after WNV Eg101 infection of tC3H/He MEFs that continued to increase through 48 h (Fig. 1B). At 48 h, 860 pg/ml of IFN beta was detected which corresponds to about 730 International units/ml based on ELISA data obtained with standard curves done on dilutions of an IFN beta sample of known unit concentration. Similar IFN levels were previously reported for WNV infected primary MEFs (Daffis et al., 2009).

The binding of IFN beta to its cell surface receptor results in activation of the Jak-STAT signaling pathway and phosphorylation of STAT1 and STAT2. A previous study showed that robust phosphorylation of STAT1 occurred in primary C3H/He MEFs after infection with WNV Eg101 (Scherbik et al., 2007). To determine whether this was also the case in transformed MEFs, activation of the Jak-STAT signaling pathway in tC3H/He MEFs by either IFN beta treatment or WNV Eg101-infection was analyzed by Western blotting. Increased levels of both total and phospho-STAT1 as well as of total and phospho-STAT2 were observed after a 3 h incubation of cells with murine IFN beta (Fig. 1C). Upregulation of total STAT1 and STAT2 levels was also observed from 12 h through 48 h after WNV Eg101 infection. Low levels of phosphorylated STAT1 (Tyr 701) were detected at 2, 6 and 12 h after infection, while robust phosphorylation was seen at 24 and 48 h. Low levels of STAT2 phosphorylation (Tyr 690) were