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A screening method for identifying disruptions in interferon signaling reveals HCV NS3/4a disrupts Stat-1 phosphorylation

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Abstract

Viruses have evolved mechanisms to inhibit the innate immune response to infection. The aim of this study was to develop an efficient screening method to identify viral proteins and their ability to block Jak–Stat signaling using hepatitis C virus (HCV) as an example. The 2FTGH cell assay system was used in combination with transient transfection of HCV proteins in this study. Using 1000 U/ml IFN and 30 mM 6-TG to treat 2FTGH cells, it was established that transient protein expression in this cell system yielded 39% and 0% cell survival for the positive (HPV E7) and negative controls (GFP expression) respectively. Transient expression of HCV Core-p7 resulted in 22% cell survival, consistent with previous reports, while expression of the HCV serine protease NS3/4a resulted in 54% cell survival. NS3/4a was subsequently shown to inhibit phosphorylation of Stat-1 at the serine residue 727. Conclusion: the 2FTGH cell assay system can be adapted for transient screening to examine the ability of viral proteins or other potential inhibitors to block the Jak–Stat signaling pathway. We show that HCV NS3/4a is able to block this pathway at the stage of Stat-1 serine 727 phosphorylation.

Keywords: Interferon; Jak–Stat; Hepatitis C virus

1. Introduction

Viral infection of mammalian cells results in activation of a number of viral recognition pathways that ultimately induce innate defences to limit viral replication. Fundamental to this antiviral response is the induction of interferon (IFN). IFNs (IFN-α and β) are essential for immune defences against viruses, in which interferon stimulated genes (ISGs) enhance the immune response and act chiefly to limit viral replication. The magnitude and breadth of the IFN response is essential in establishing both early and adaptive immune responses. IFNs act to limit viral replication and infection via rapid activation of the Jak–Stat pathway, which culminates in the transcription of hundreds of ISGs. In turn, viruses have developed mechanisms to target the upregulation of ISGs in infected cells, including inhibition of toll-like receptors (TLRs), the action of interferon regulatory factor 3 (IRF3), induction of interferon synthesis, the signal transduction pathway and the functions of ISGs such as RNA-activated protein kinase (PKR) and 2–5 oligoadenylate synthetase (Katze et al., 2002). Furthermore many viruses have evolved mechanisms that target more than one part of this system, indicating that blocking activation of IFN and ISGs within virally infected cells is crucial for viral replication.

Central to the antiviral action of IFN, the Jak–Stat signal transduction pathway is one of the most targeted host innate immune responses by viral proteins. Following viral infection of a cell, IFN-β is produced through the activation of dsRNA recognition pathways (TLR3 and RIG-I), leading to the activation of the IFN-α/β receptor in either an autocrine or paracrine manner. This interaction results in the induction of signal transduction through the Jak–Stat pathway. Upon IFN receptor binding and activation, the receptor-associated tyrosine kinase 2 (TYK2) is phosphorylated by Janus Kinase I (JAK1), which is then cross-phosphorylated (Constantinescu et al., 1994). This results in the phosphorylation of signal transducer and activator of transcription 2 (Stat-2) and its subsequent activation of Stat-1 (Li et al.,

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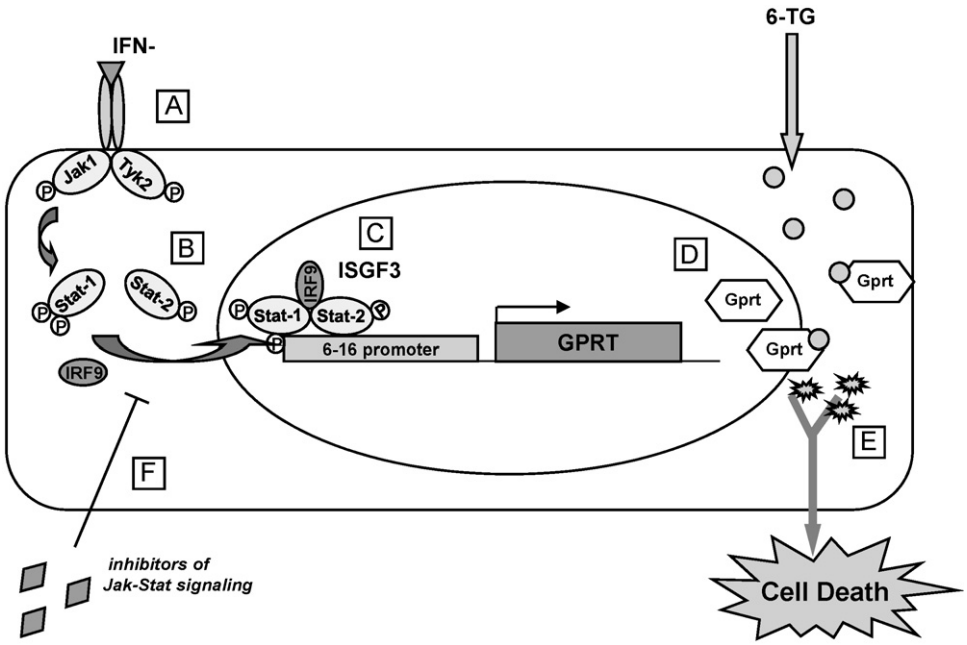

Fig. 1. The 2FTGH cell system. 2FTGH cells are treated with IFN-α, which binds (A) to the IFN receptor and triggers cross-phosphorylation of Jak1 and TYK2, this leads (B) to the phosphorylation of Stat-1 and Stat-2 which then complexes with IRF-9 to form ISGF3 (C) and translocate to the nucleus where it is able to bind to the ISRE transcription site on the 6–16 promoter and (D) drive production of GPRT. GPRT is able to metabolise 6-TG which is added to the cells, to form toxic guanine analogues which result in cell death (E). However, if viral proteins able to block parts of the Jak–Stat signaling cascade are transiently transfected into 2FTGH cells (F), there will be a reduced production of GPRT, and increased cell survival will be seen.

1996; Yan et al., 1996). The Stat-1/2 heterodimer is then able to disassociate from the receptor and bind to IRF-9 forming the transcription factor IFN-stimulated gene factor 3 (ISGF-3) (Fu et al., 1992; Schindler et al., 1992). ISGF3 then translocates to the nucleus and is able to bind to the IFN-stimulated response element (ISRE) of multiple genes (Dale et al., 1989; Levy et al., 1989), leading to the transcription of thousands of ISGs, including classical ISGs such as ISG56, ISG15 and RANTES (Der et al., 1998; Grandvaux et al., 2002).

A number of viruses have evolved mechanisms to inhibit Jak–Stat signaling. For example, Japanese encephalitis virus (JEV) inhibits tyrosine phosphorylation of the IFNα/β receptor-associated TYK2 (Lin et al., 2006a, 2004). Langat virus (LGTV), a member of the tick-borne encephalitis family blocks activation of Jak1 and TYK2 (Best et al., 2005). The adenovirus E1A protein inhibits IFN signaling by blocking the formation of the ISGF3 complex (Kalvakolanu et al., 1991), as does the human papillomavirus E7 protein, which competitively binds IRF-9 (Barnard and McMillan, 1999; Barnard et al., 2000). West Nile virus (WNV) has also been shown to block the Jak–Stat pathway at the level of Stat-1 and/or Stat-2 phosphorylation, with the non-structural proteins NS2a, 2b, 3, 4a and 4b being involved (Liu et al., 2005). A number of these viruses are major pathogens in humans, including JEV and WNV, both members of the *flaviviridae* family. Another member of this family, HCV is also a significant human pathogen worldwide, that results in a persistent infection in approximately 70% of infected individuals. The ability of HCV and other pathogenic viruses to block the Jak–Stat pathway, and an understanding of the mechanisms utilised for inhibition of interferon signalling may offer further targets for drug discovery. It was the aim of this study to further develop an efficient screening assay based on the 2FTGH cell system to identify individual viral proteins capable of inhibiting the Jak–Stat signaling pathway, using HCV as a model virus.

2. Materials and methods

2.1. 2FTGH cell line

The human fibrosarcoma 2FTGH cell line was kindly provided by G Stark (Cleveland Centre for Structural Biology, OH, USA)(Pellegrini et al., 1989a) and was maintained in Dulbecco's modified Eagle medium (Gibco-BRL, Grand Island, NY) supplemented with 10% fetal bovine serum, L-glutamine, penicillin and streptomycin in a humidified 37 °C/5% CO_2 incubator. 2FTGH cells were placed under the selective pressure of hygromycin (250 ng/ml, Sigma, MO, USA) every second passage, and were seeded for experiments following a single cell split into hygromycin free media. 2FTGH cells (Pellegrini et al., 1989b) are stably transfected with the interferon sensitive 6–16 promoter, which drives the expression of the *E. coli* guanine phosphoribosyltransferase (GPRT) gene. This cell line can be utilised to select for the loss of IFN sensitivity in the presence of the compound 6-thioguanine (6-TG), as GPRT metabolises 6-TG to form the toxic analogue guanine, making cell death the assay end point (Fig. 1).

2.2. Quantification of viable 2FTGH cells

2FTGH cells were seeded in 24-well plates at 0.5, 1, 2, 4 and 8×10^4 cells per well and left to attach overnight. The following day cell culture media was removed, and the cells fixed in either 200 μl of cold acetone/methanol (1:1) at −20 °C for 10 min or left unfixed before being rinsed three times with 1× PBS. Cells fixed in acetone/methanol were then stained with 0.1% crystal violet for 1 min and rinsed with 1× PBS, while unfixed cells were incubated in 100 μl of 2 M NaOH for 20 min and total protein analysed using the BioRad protein assay and a MR5000 plate reader (Dynatech, VA, USA). Leeching of crystal violet for quantitative purposes was performed by the addition of 600 μl of 10% acetic acid or 1% SDS. The plates were shaken for 30 min and absorbance values of 50 μl aliquots read at 570 nm on an MR5000 plate reader. Cells fixed prior to crystal violet staining were also scanned on a HP Scanjet 7400c using HP Precision Scan Pro 3.01, before densitometry analysis using ImageQuant 5.2 (Molecular Dynamics, Sunnyvale, CA, USA). Cell survival was expressed as a percentage of the test well densitometry units divided by the mean of the control wells (containing either no IFN or no 6-TG), and graphed as the mean value plus standard error of the mean. Experiments to determine the best enumeration method were performed in duplicate.

2.3. 2FTGH cell assay

2FTGH cells were seeded at 2×10^4 cells per well of a 24-well plate and left to attach for 6 h before being transfected with 400 ng of plasmids expressing the following; pE-GFP-N1 (BD biosciences, NJ, USA), pEF.1E7 (a kind gift from Dr N. McMillan, University of Queensland, Australia) or HCV expression plasmids (pCDNA3.1-C-P7, NS3, NS4a; pCMV-NS4b, NS5a, NS5b; pCDNA6-NS3/4a, NS3-5B, all kind gifts from Dr K. Li, University of Texas Medical Branch, USA) using Fugene as per manufacturers instructions (Roche, IN, USA). 24 h after transfection the culture media was replaced with media containing 0, 50, 200 or 1000 units/ml IFN-α2b (Schering Plough, NJ, USA) alone or in combination with 10, 20 or 30 μM 6-TG (Sigma, MO, USA). Cells were then left for 72 h before the media was removed and cells were fixed and stained as mentioned previously. All experiments were performed a minimum of three times.

2.4. ISRE reporter constructs

2FTGH cells were plated at 8×10^4 cells per well in a 12-well plate, and allowed to settle overnight before transfection with 400 ng p(9-27ISRE)4tkΔ(-39)Lucter reporter plasmid (a kind gift from Dr R.E. Randall, Division of Basic Medical Sciences, St. George's, University of London) and 400 ng of either pCDNA3.1-NS3, NS4a or pCMV-NS5b or pCDNA6-NS3/4a. Cells were also co-transfected with 10 ng PhRL-TK (a renilla expressing plasmid; Promega, WI, USA) to control for variations in transfection efficiency. Following transfection cells were allowed to incubate for 24 h before being treated with 1000 U/ml IFN-α for 5 h. Luciferase activity was measured using the luciferase assay system (Promega) and a Turner TD 20/20 luminometer. All experiments were performed in triplicate.

2.5. Analysis of Stat-1 expression in 2FTGH cells

2FTGH cells were plated at 2×10^6 cells per well of a 6-well plate, and allowed to settle overnight before transfection with 2 μg of either pE-GFP-N1, pCDNA6-NS3/4a or pCDNA-3-5b. Twenty four hours following transfection cells were incubated with 150 units/ml of IFN-α2b for 30 min and the protein extracted and separated by SDS PAGE and transferred to nitrocellulose as previously described (Helbig et al., 2005). Membranes were blocked with 5% skim milk and incubated overnight with a 1/1000 dilution of anti-rabbit phospho Stat-1 Tyr701, phosphor Stat-1 Ser-727, Stat-1 antibody (Cell Signaling, MA, USA) or a 1/1000 dilution of anti-mouse β-actin (Sigma). Membranes were then incubated for 1 h at RT in either a 1/2000 dilution of an anti-rabbit-HRP antibody (Cell Signaling) or a 1/10,000 dilution of anti-mouse HRP antibody (Rockland, Gilbertsville, PA). Protein bound to antibody was visualized by chemiluminescence (ECL, Amersham Biosciences, Piscataway, NJ).

3. Results

3.1. Optimization of 2FTGH cell viability measurements

The 2FTGH cell assay is based on the activity of the highly IFN sensitive 6–16 promoter to drive expression of GPRT, which in the presence of the compound 6-TG results in cell death. To use this system in an assay to identify viral proteins that disrupt Jak–Stat signaling, it was necessary to develop a reproducible technique for enumeration of cell viability. To achieve this 2FTGH cells were plated at various concentrations and incubated overnight before being treated in various fashions to enumerate viable cells. Initial studies involved staining the cells (fixed or unfixed) with crystal violet for 30 min followed by recovery of cell associated crystal violet with either acetic acid or SDS. As can be seen in Fig. 2A, 1% SDS treatment of unfixed cells gave the best correlation between cell number and absorbance reading with an r^2 value of 0.97. Fixing of the cells before crystal violet staining appeared to decrease the ability of the more confluent cell wells to liberate crystal violet into the SDS mixture, with decreased absorbance readings given for 8×10^4 cells compared to 4×10^4 cells in both the SDS and acetic acid treatments where the cells were fixed prior. Total protein extraction of the cells was also analysed as an enumeration tool of cell viability (Fig. 2B) giving an r^2 value of 0.99, however, this technique does not allow prior staining of the cells for visualization of cell viability. The technique found to be most useful was densitometry of the scanned plate (following cell fixation and crystal violet staining) using ImageQuant. As can be seen in Fig. 2B this method gave an r^2 value of 0.98, and was the preferred technique over SDS leeching of the crystal violet on unfixed cells, given the difficult nature of handling unfixed cells. The scanning densitometry

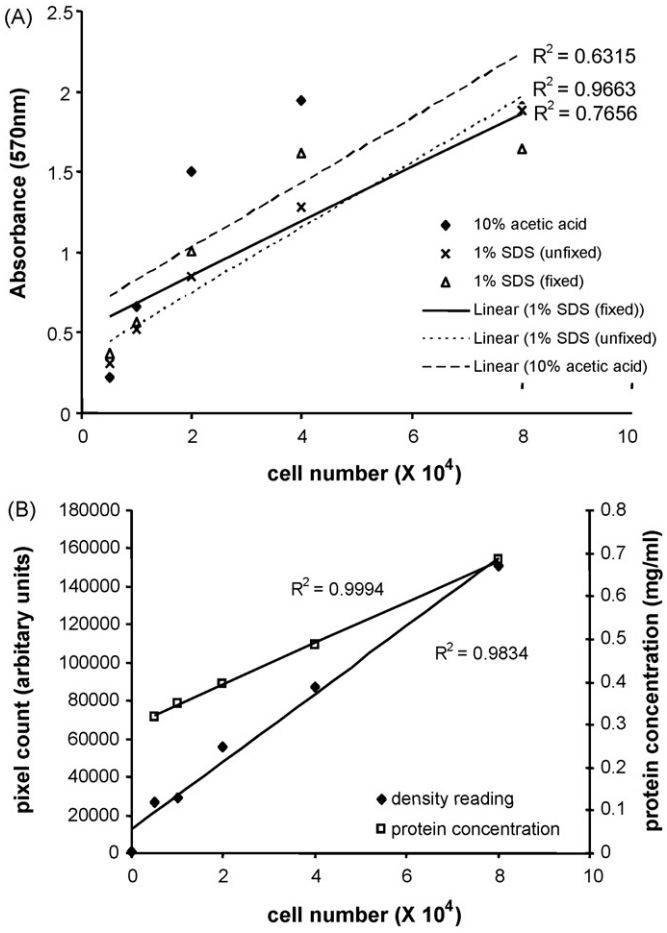

Fig. 2. Optimisation of 2FTGH cell viability measurements. (A) 2FTGH cells were seeded at varying concentrations, allowed to settle for 24 h before being stained with crystal violet (fixed or unfixed). The stained 2FTGH cells were then immersed in either 10% acetic acid or 1% SDS for 30 min before absorbance readings were taken at 600 nm. (B) 2FTGH cells were seeded as above and either fixed in methanol, stained with crystal violet, scanned and densitometry readings performed utilising Image Quant 5.2 or cells were protein extracted and assayed for protein concentration.

method was therefore subsequently used for the remainder of this study.

3.2. Assessment of the 2FTGH transient system to demonstrate Jak–Stat pathway interference

2FTGH cell death only occurs in the presence of both 6-TG and IFNα, assuming there is no disruption to the Jak–Stat pathway. A titration assay using varying concentration of both compounds was utilised on 2FTGH cells plated 24 h prior. Fig. 3A and B demonstrate that the cells undergo varying degrees of survival under the influence of these two compounds, however, in the absence of either 6-TG or IFN-α there is complete cell survival. The full effects of the guanine toxic analogue appear to be realized only at 1000 U/ml of IFN, and in the presence of all concentrations of 6-TG, where we see negligible cell survival.

Next we assessed if this assay could be adapted to transient expression of viral proteins as previous studies have relied on stable expression of the protein of interest (Clarke et al., 2004). Stable expression is time consuming and not amenable to high-throughput screening. 2FTGH cells were initially transiently transfected with the E-GFP expression plasmid at various concentrations and assessed for transfection efficiently 24 h post-transfection using immunofluoresence. Cell counting was able to demonstrate that greater than 95% of cells were expressing GFP and that this was unrelated to either cell density or plasmid concentration (data not shown). To determine the feasibility of a transient protein expression approach, 2FTGH cells were transiently transfected with the mammalian expression vector pEF.1E7, which expresses the human papillomavirus protein E7, a known inhibitor of the Jak–Stat signaling pathway (Barnard and McMillan, 1999). In the presence of the negative control, E-GFP, there is negligible cell survival (less than 10%) at concentrations of IFN at or above 200 U/ml (Fig. 4A and B), regardless of the 6-TG concentration, with cell survival observed in the wells containing 50 U/ml of IFN likely to be due to inefficient production of GPRT in the system. However, in the presence of HPV protein E7, 2FTGH cell survival rates are 54% and 39% in presence of 1000 U/ml of IFN and 10 and 30 mm 6-TG, respectively (Fig. 4B). This is indicative that a transient expression approach of viral proteins in the 2FTGH cells is able to identify inhibitors of the Jak–Stat pathway.

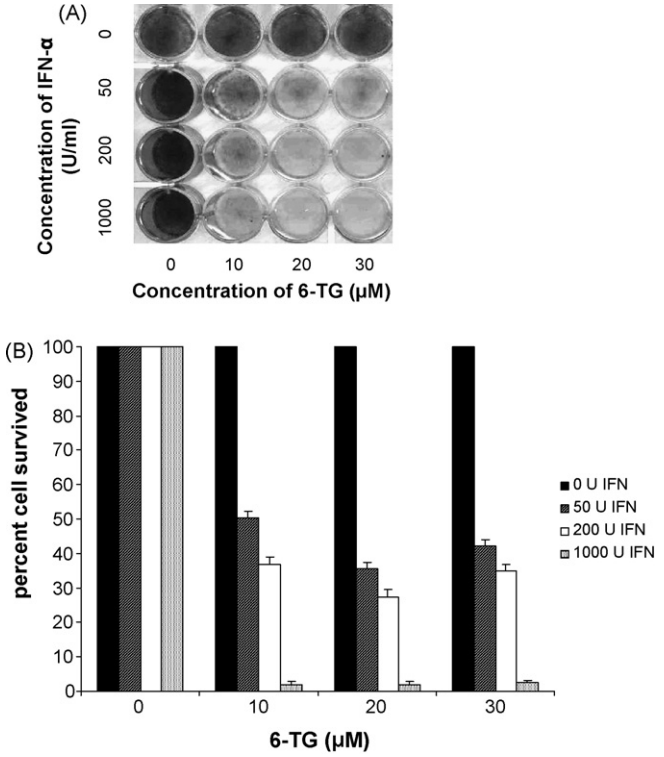

Fig. 3. IFN-α/6-TG titration on 2FTGH cells. 2FTGH cells were treated with varying concentrations of IFN-α and 6-TG, left for 3 days and then fixed and stained with crystal violet before. (A) Cell culture plates were scanned and (B) densitometry readings performed using Image Quant 5.2.

3.3. HCV proteins are able to block the Jak–Stat pathway

Recently it has been shown that HCV can block various arms of the double stranded RNA signaling cascade resulting

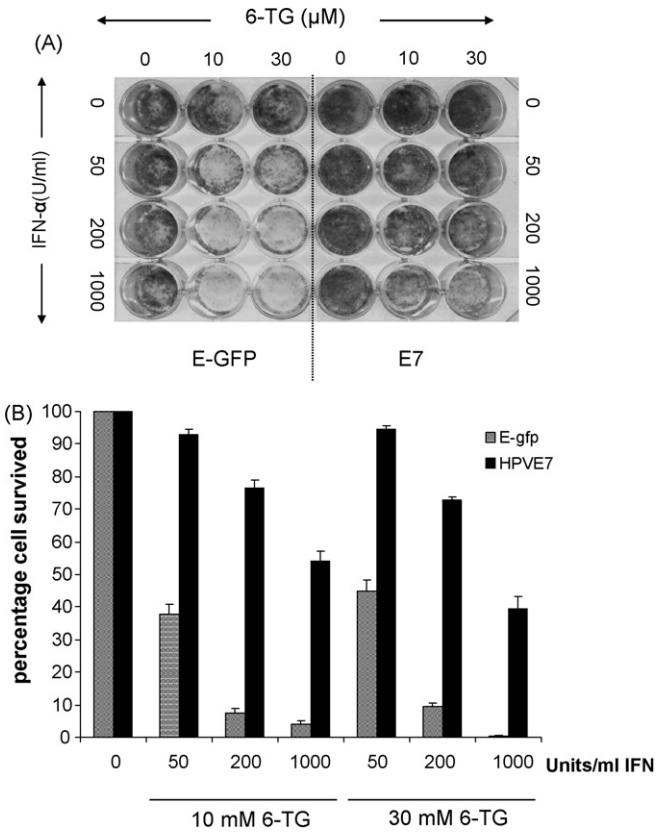

Fig. 4. IFN-α/6-TG titration on 2FTGH cells using HPV E7. 2FTGH cells were transfected with either HPV E7 or E-GFP and then treated for 3 days with varying concentrations of IFN-α and 6-TG, before being fixed and stained with crystal violet. (A) Cell culture plates were scanned and (B) densitometry readings performed using Image Quant 5.2.

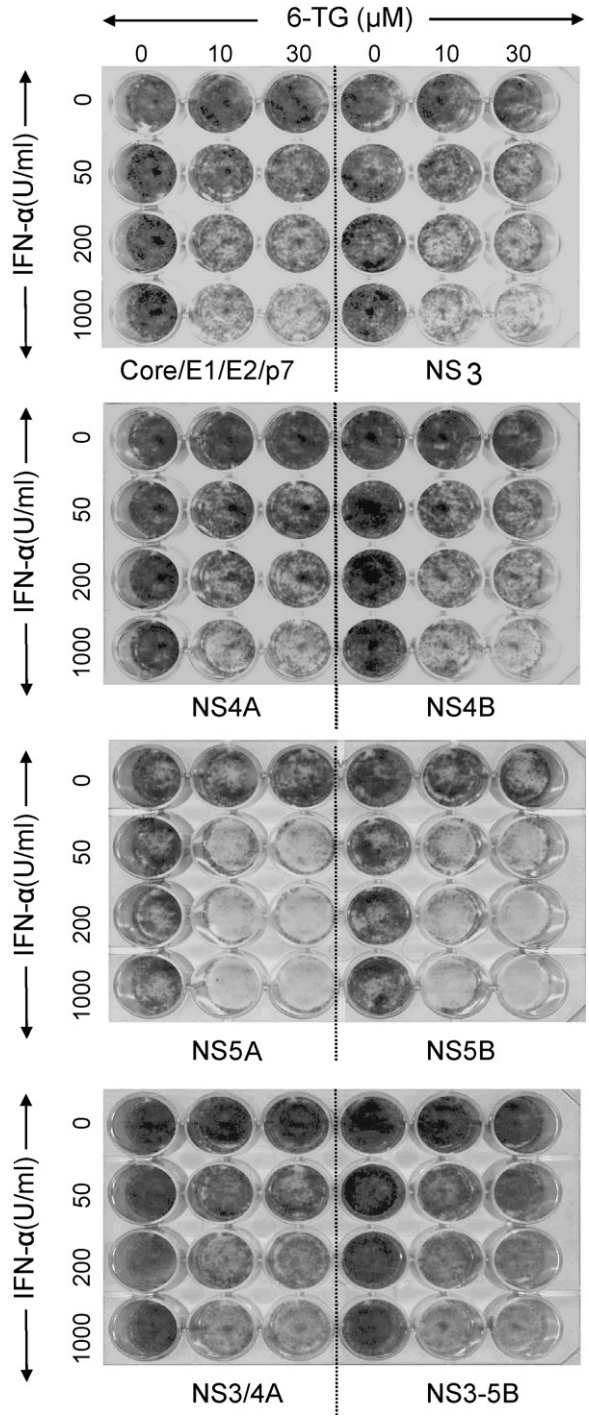

Fig. 5. Analysis of individual HCV proteins ability to inhibit the Jak–Stat pathway. 2TGH cells were transiently transfected with HCV C-P7, NS3, NS4a, NS4b, NS5a, NS5b, NS3/4a and NS3-5b. 24 h following transfection cells were treated with 50, 200 or 1000 U/ml IFN-α and either 10 or 30 μM 6-TG for 3 days, before being fixed and stained with crystal violet.

in a block of IRF3 phosphorylation (Foy et al., 2003; Li et al., 2005). In contrast, the ability of HCV to block the Jak–Stat signalling pathway is relatively unexplored, although previous reports have suggested that HCV core protein is able to inhibit Jak–Stat signalling via blocking Stat-1 phosphorylation (Lin et al., 2005, 2006b). We therefore sought to investigate the ability of all HCV proteins to block the Jak–Stat signaling pathway using the 2FTGH cell system mentioned above in a transient manner. As can be seen in Fig. 5, 2FTGH cells transfected with the HCV core-p7 expressing construct resulted in significant cell survival, with 38% and 23% of cells surviving in the presence of 1000 U/ml of IFN and 10 and 30 mM 6-TG, respectively ($p < 0.05$, Fig. 6). This result was further validation of the transient 2FTGH assay, given the previous literature reports mentioned above. Expression of HCV NS3, NS4a and NS4b all demonstrated an ability to inhibit the Jak–Stat pathway at high concentrations of IFN, and low concentrations of 6-TG (29%, 32% and 27%, respectively, $p < 0.05$), however, this effect was diminished at the higher 30 mm 6-TG concentration for NS3 and NS4B (7% and 3%, respectively, $p > 0.05$). In contrast, HCV NS4A protein with 32% and 25% cell survival seen at the highest IFN concentration, and 10 and 30 mM 6-TG, respectively demonstrated an ability to abrogate Jak–Stat signaling ($p < 0.05$). HCV NS5a and NS5b displayed no inhibition of the Jak–Stat signaling pathway (Figs. 5 and 6). Expression of NS3/4a and NS3-5b in the 2FTGH cell system demonstrated significant cellular survival rates of 58% and 51% at the highest IFN and lowest 6-TG concentration, respectively, and 54% and 45% at the highest IFN and 6-TG concentrations ($p < 0.05$). These results strongly suggest that the NS3/4a protein complex has an

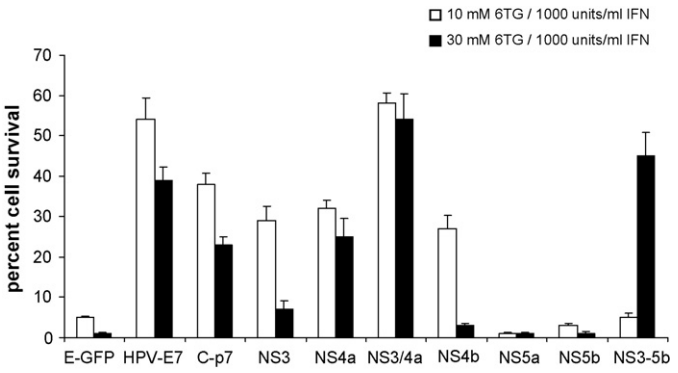

Fig. 6. HCV C-P7 and NS3/4a are able to inhibit the Jak–Stat pathway. 2FTGH cells were transiently transfected with individual HCV protein expression plasmids and then treated with 1000 U/ml IFN-α and either 10 or 30 μM 6-TG before being fixed, stained with crystal violet, scanned and densitometry readings performed.

ability to block Jak–Stat signaling. Interestingly this inhibition was consistently greater than that seen for our positive control HPV E7.

In an attempt to further corroborate the ability of NS3/4a to inhibit Jak/Stat signaling, 2FTGH cells were co-transfected with an ISRE reporter plasmid in combination with either the NS3/4a, NS3, NS4a or NS5b expression plasmid. Following treatment with IFN-α, a significant decrease in promoter activity was seen in the presence of NS3, NS4a and NS3/4a ($p < 0.05$, Fig. 7). No decrease in promoter strength was seen in the presence of NS5b or for the expression of any HCV proteins on activity of the GAS reporter plasmid (results not shown). These results further establish a role for HCV NS3/4a in limiting the Jak/Stat signaling pathway.

3.4. HCV NS3/4a is able to block Stat-1 serine phosphorylation

In an attempt to elucidate the mechanism of inhibition of Jak–Stat signalling by HCV NS3/4a, we examined Stat-1 phosphorylation. 2FTGH cells were transiently transfected with NS3/4a and NS5b (as a negative control), before being treated

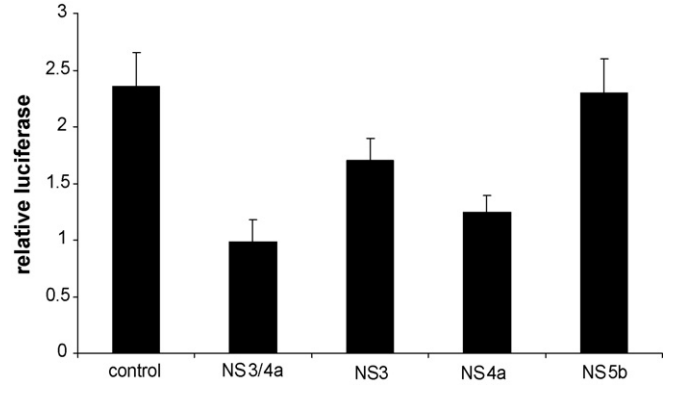

Fig. 7. HCV NS3/4a is able to restrict ISRE activity. 2FTGH cells were transiently transfected with individual HCV expression plasmids in combination with an ISRE driven luciferase reporter plasmid. Cells were treated with 1000 U/ml IFN-α for 5 h before lysates were analysed for luciferase activity.

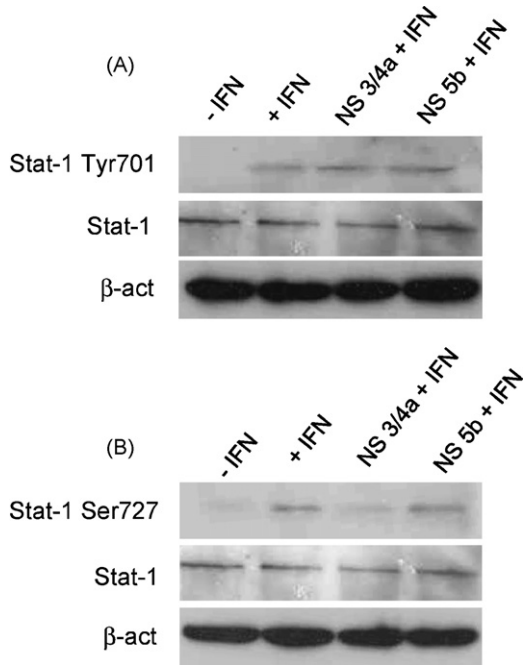

Fig. 8. HCV NS3/4a inhibits Stat-1 Ser-727 phosphorylation. 2FTGH cells were transiently transfected with either HCV NS3/4a, NS5b or empty vector before being stimulated with 150 U/ml IFN-α for 30 min. Cellular protein was extracted, and western blot analysis utilised to visualise phosphor-Stat-1 Tyr701, Ser-727, Stat-1 and β-actin.

with IFN-α to induce Stat-1 phosphorylation. 2FTGH cells with no IFNα stimulation displayed no Stat-1 phosphorylation at tyrosine 701(Fig. 8a), while a low level background was noticeable for Stat-1 serine 727 phosphorylation (Fig. 8b). As expected, stimulation with IFN-α resulted in phosphorylation at tyrosine 701 and serine 727 residues, however, this effect was abrogated in the presence of NS3/4a overexpression for serine 727 phosphorylation (Fig. 8b) but not 701 phophorylation. No changes were evident in unphosphorylated Stat-1 in the presence of either HCV NS3/4a or NS5b. These results are suggestive for the first time that HCV NS3/4a is able to inhibit Jak–Stat signalling by decreasing Stat-1 serine 727 phosphorylation in the presence of IFN-α.

4. Discussion

The induction of IFN-α is a powerful host defence mechanism against viral infection, and many viruses have evolved strategies to overcome the antiviral effects of IFN. Interferon exerts its antiviral effects by inducing the expression of hundreds to thousands of ISGs, many of which have been shown to be antiviral. Central to IFN expression of ISGs is the Jak–Stat signalling pathway and hence many viruses target this pathway to evade the effects of IFN. Thus, identification of viral proteins that block the action of IFN is essential to our understanding of virus/host interactions and pathogenesis, not to mention potential targets for therapeutic action. This study outlines a screening method for identifying the ability of viral proteins to block the Jak–Stat signalling pathway using the 2FTGH cell system (Clarke et al., 2004; Pellegrini et al., 1989a). This cell system

utilised in combination with IFN-α stimulation and 6-TG offers the ability to test if a transiently expressed viral protein(s) is able to block the activation of the tightly IFN regulated 6–16 promoter (Ackrill et al., 1991; Chernajovsky and Kirby-Sanders, 1990; Kelly et al., 1985). This cell line has been used extensively to investigate IFN signaling but has been relatively under utilised to study the effect of viral protein expression on Jak–Stat signal transduction.

Utilising the E7 protein of HPV, a known inhibitor of the Jak–Stat pathway (Barnard and McMillan, 1999), we have been able to demonstrate that the 2FTGH cell system can be used in a transient assay to investigate viral protein inhibition of the Jak–Stat signaling pathway. Specifically we have shown that 2FTGH cells transiently expressing HPV E7 followed by treatment with IFN-α and 6-TG resulted in cell survival compared to complete cell death in cells expressing E-GFP, a protein that does not inhibit Jak–Stat signaling. This system was optimised further to enable numerical outputs of cell survival using densitometry readings.

Utilising this transient approach we were able to assess the ability of individual HCV proteins to block the Jak–Stat pathway. Expression of the full HCV polyprotein has previously been shown to inhibit the Jak–Stat pathway downstream of Stat-1 tyrosine phosphorylation (Heim et al., 1999), and HCV core protein has also been demonstrated to block Stat-1 tyrosine phosphorylation (Bode et al., 2003), however, little work has been performed on the ability of the remainder of the individual HCV non-structural proteins to inhibit the Jak–Stat pathway. This study was able to demonstrate that at the highest level of IFN-α stimulation and 6-TG treatment HCV C-p7, NS4a, NS3/4a and NS3-5b were all found to inhibit the Jak–Stat pathway, displaying cell viabilities of 22%, 25%, 54% and 46%, respectively. NS3 alone showed minimal ability to inhibit the system, but a synergistic increase in cell survival was seen with the combination of NS3/4a, with the cell survival of 54% being higher than that of the E7 control at 39%. Consistent with a previous observation, HCV core (Lin et al., 2005, 2006b) also blocked Jak–Stat signaling.

The ability of HCV NS3/4a to block the Jak–Stat pathway was further examined utilising Western blotting techniques to visualise Stat-1 phosphorylation following IFN-α treatment of the 2FTGH cells. Stat-1 Ser-727 phosphorylation was inhibited in the presence of NS3/4a. Ser-727 phosphorylation of Stat-1 is necessary for ISGF3 transcriptional activation (Pilz et al., 2003), however, identification of the serine kinase(s) that are involved is still undetermined. It has been demonstrated that the MAP kinases, including MEK1, ERKs, JNKs, MSK1 and in particularly p38 MAP kinase activity are required for the serine phosphorylation of Stat-1. Inhibition of p38 kinase activity abolishes Stat-1 Ser-727 phosphorylation by IFN-α, but the different kinetics of p38 activation and Ser-727 phosphorylation still suggest an indirect role for p38 (Goh et al., 1999; Zhang et al., 2004). The vaccinia virus M2L protein has been shown to inhibit ERK2 activation, and the conserved alphaherpesvirus structural protein Us2 has recently been demonstrated to block nuclear localisation of ERK2 by redistributing it to the plasma membrane and to a perinuclear vesicular compartment (Gedey et al., 2006; Lyman et al., 2006). It is possible that HCV NS3/4a somehow interferes with the MAP kinase signaling cascade resulting in decreased Stat-1 Ser-727 phosphorylation; however, this is still to be determined.

This is the first time to our knowledge that NS3/4a has been shown to inhibit Stat-1 phosphorylation. Previously NS3/4a has been shown to degrade Stat-1 protein (Lin et al., 2005), however, this was not seen in our system. HCV NS3/4a is the viral protease responsible for cleavage of the HCV non-structural proteins, with NS3 showing protease and helicase activity and NS4a being a necessary co-factor. NS3/4a has previously been shown to cleave components of the double stranded RNA signaling pathway TRIF and Cardif, resulting in a block in IRF-3 phosphorylation, and an eventual reduction in antiviral ISG formation (Li et al., 2005; Meylan et al., 2005). These observations coupled with our study suggest that NS3/4a may act at multiple points in innate immune pathways.

This study has described a high throughput method for the determination of the ability of individual viral proteins to inhibit the Jak–Stat pathway, utilising the 2FTGH cell system. The ability of pathogenic viruses to block the Jak–Stat pathway, and an understanding of the mechanisms utilised for inhibition of interferon signaling by these viruses may in the future identify therapeutic strategies.

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