Inhibition of vesicular stomatitis virus infection in epithelial cells by alpha interferon-induced soluble secreted proteins

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Interferons (IFNs) are potent antiviral cytokines that inhibit infection by a wide spectrum of viruses by activating the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway. Several IFN-induced antiviral proteins including 2′,5′-oligoadenylate synthetase, dsRNA-activated protein kinase and Mx play a critical role in conferring the antiviral properties of IFN. However, studies have shown that additional antiviral factors are involved in addition to these proteins during IFN-mediated antiviral action. In an effort to characterize these novel antiviral factors, the antiviral mechanism of alpha IFN (IFN-α) against vesicular stomatitis virus (VSV) was investigated in human lung epithelial A549 cells. These studies demonstrated that soluble secreted antiviral proteins as the constituents of conditioned medium prepared from IFN-α-treated cells reduced VSV infectivity by more than 2 logs, compared with a 4 log inhibition observed following treatment of cells with IFN-α. The antiviral mechanism of these secreted proteins appeared to act at the level of cellular entry of VSV. Interestingly, the IFN-α-induced antiviral proteins were secreted independently of STAT1 (an essential component of the JAK/STAT pathway), demonstrating that the release of such extracellular soluble antiviral proteins from cells may represent an alternative mechanism of the antiviral defence strategy of IFN towards VSV infection.

INTRODUCTION

Interferons (IFNs) constitute an important element of the innate immune response elicited by the host to control virus infection (Stark et al., 1998; Biron & Sen, 2001; Bose & Banerjee, 2003). IFNs are classified into two classes, namely IFN I and IFN II, representing IFN-α/β and IFN-γ, respectively. The former is produced from non-immune cells (e.g. epithelial cells), whilst the latter is restricted to immune cells. IFN-α/β induces antiviral activity by binding to IFN receptors, which results in activation of Janus kinases (JAKs) and signal transducers and activators of transcription (STATs) (Stark et al., 1998; Biron & Sen, 2001) and formation of the IFN-stimulated gene factor 3 (ISGF3) complex comprising STAT1, STAT2 and p48. The ISGF3 complex translocates to the nucleus to induce a wide variety of IFN-stimulated genes, some of which possess antiviral activity. The three well-established antiviral proteins involved in IFN-mediated inhibition of virus infection are: (i) the 2′,5′-oligoadenylate synthetase (OAS)/RNaseL pathway (Silverman, 1997), which degrades viral RNAs; (ii) the dsRNA-activated protein kinase (PKR), which inhibits mRNA translation (Clemens & Elia, 1997); and (iii) the Mx proteins (Mx1 in mice and MxA in humans), which possess GTPase activity and which restrict virus infection at several stages of the viral life cycle (Haller & Kochs, 2002). These three IFN-induced proteins restrict a wide spectrum of viruses (Staeheli & Pavlovic, 1991; Zhao et al., 1996; Stojdl et al., 2000; Behera et al., 2002), including negative-sense, non-segmented, single-stranded RNA (NNS) viruses.

NNS viruses are enveloped viruses that undergo replication/transcription in the cytoplasm. Representative NNS viruses include vesicular stomatitis virus (VSV), human parainfluenza virus type 3 (HPIV-3) and human respiratory syncytial virus (Lamb & Kolakofsky, 2001; Rose & Whitt, 2001). Although the three IFN-induced proteins described above are capable of restricting NNS virus infection following IFN treatment, several studies have shown that alternative IFN-induced factors are also operative in IFN-treated cells. For example, it has been shown that IFN elicits its antiviral activity against HPIV-3 independently of PKR,
Mx and OAS/RNaseL (Choudhary et al., 2001). The ability of IFN to inhibit virus replication by novel antiviral pathways has also been observed for VSV. Studies with PKR, Mx and RNaseL triple-knockout mouse embryonic fibroblasts have revealed the ability of IFN to restrict VSV infection in the absence of the these three IFN-induced proteins (Zhou et al., 1999). A similar study also reported the ability of IFN to restrict VSV replication in cells expressing PKR, OAS/RNaseL and Mx proteins (Pattyn et al., 1999). Moreover, phospholipid scramblase has been identified as a candidate IFN-induced antiviral protein that restricts VSV infection (Dong et al., 2004). Thus, IFN has been shown to induce novel antiviral factors that restrict virus replication.

In an effort to characterize these novel IFN-induced factors, we investigated the mechanism of antiviral function of IFN-α (henceforth designated IFN) in human lung epithelial A549 cells. We chose VSV for our studies, as VSV is highly sensitive to IFN and is routinely used to assay the antiviral activity of IFN (Marcus & Sekellick, 1978). We also utilized epithelial cells for our studies, as these cells serve as the primary site for productive infection of several NNS viruses (Garofalo & Haeberle, 2000) and therefore studies on the IFN-dependent innate antiviral response by epithelial cells are important to elucidate host defence mechanisms. Our studies revealed that IFN activates its antiviral function as early as 1 h after treatment of cells and that this activity is maintained during infection (in the absence of IFN added exogenously during infection). Moreover, we demonstrated that IFN induces heat- and acid-sensitive soluble secreted antiviral proteins that inhibit VSV entry. Surprisingly, the induced secreted antiviral proteins were produced independently of the JAK/STAT signalling pathway. Our studies have thus elucidated (i) the role of extracellular IFN-induced soluble secreted proteins as opposed to cytoplasmic proteins such as PKR, OAS/RNaseL and Mx in conferring antiviral activity, and (ii) the existence of a putative JAK/STAT-independent signalling pathway/mechanism for the antiviral function of IFN.

METHODS

Virus and cells. VSV (Indiana serotype, Mudd–Summers strain) was propagated in BHK-21 cells (Bose et al., 2003b; Dong et al., 2004). Human lung epithelial A549 cells, African green monkey kidney epithelial Vero cells and human fibrosarcoma 2TGH and U3A cells were maintained in DMEM (Gibco-BRL) supplemented with 10% FBS. The viral titer was monitored by plaque assay using CV-1 (African green monkey fibroblasts) and L929 (mouse fibroblasts) cells (Bose & Banerjee, 2002; Bose et al., 2001, 2003a, b, 2004). In our studies, similar results were obtained when plaque assays were performed using either CV-1 or L929 cells (data not shown). The plaque assay values given in the figures are expressed as p.f.u. ml⁻¹ and represent the mean ± SD of three independent determinations.

Western blot analysis. Western blot analysis was performed essentially as described previously (Bose et al., 2003b) using polyclonal anti-VSV P protein antibody.

Preparation of conditioned medium. In order to prepare control mock-conditioned medium (MCM) and IFN-α-conditioned medium (IFN-CM), confluent cells were either left untreated or incubated with IFN-α (1000 U ml⁻¹; Sigma-Aldrich) for 1–2 h. After six washes in PBS, fresh medium (DMEM/10% FBS) was added to the cells. Following incubation at 37 °C for 24 h, the medium collected from these cells was centrifuged at 800 g for 10 min and the supernatant (MCM or IFN-CM) was collected and applied to fresh cells to examine the effect of IFN-induced soluble factors on VSV infection.

VSV infection following IFN and IFN-CM treatment. To study the antiviral function of IFN and IFN-CM, cells were pre-treated with IFN (1000 U ml⁻¹) or IFN-CM for 0.5–18 h. Following incubation, VSV (m.o.i. of 0.2) was added to the washed cells and allowed to adsorb for 1.5 h at 37 °C in the presence or absence of IFN or IFN-CM. Cells were washed to remove unbound virus and infection was allowed to continue for an additional 36 h in the presence or absence of IFN or IFN-CM. Infected cell lysates were used for Western blot analysis with anti-VSV P protein antibody. In addition, culture supernatants were collected to measure virus yield by plaque assay.

ELISA to detect IFN-α and IFN-β in MCM and IFN-CM. MCM and IFN-CM derived from A549 and Vero cells along with IFN-α and IFN-β (10 and 100 U) added exogenously to the conditioned media and DMEM were analysed using ELISA kits (PBL Biomedical Laboratories) to detect IFN-α and IFN-β. The ELISA was performed according to the manufacturer’s specification and the results were expressed as pg IFN (5 pg = 1 U).

Treatment of IFN-CM with neutralizing antibodies against IFN-α/β. MCM and IFN-CM derived from A549 cells were either left untreated or incubated with either IFN-α- or IFN-β-neutralizing antibodies (1000 neutralizing U; Biosource International) for 6 h at room temperature. Similarly, 100 or 100 U IFN-α or IFN-β ml⁻¹ added exogenously to DMEM was incubated with the corresponding neutralizing antibodies. The medium was then added to A549 cells for pre-treatment (8 h for the conditioned media, 16 h for DMEM plus IFNs), followed by infection with VSV. The viral titre was determined at 36 h post-infection (p.i.). In one set of experiments, A549 cells were only pre-treated with neutralizing antibodies prior to virus infection.

Immunofluorescence analysis. A549 cells grown on coverslips were either left untreated or incubated with IFN-α (1000 U ml⁻¹) or CM for 8 h at 37 °C. Cells were washed twice with PBS and VSV (m.o.i. of 10) was added in the presence of 100 µg cycloheximide ml⁻¹. After adsorption for 45 min, the cells were washed and fresh medium was added. At 45 min p.i., cells were fixed with 3.7% formaldehyde in PBS and stained with anti-VSV P antibody, followed by FITC-conjugated anti-rabbit IgG. Cells were visualized under a Leica CSM confocal laser-scanning microscope (Bose et al., 2001; Bose & Banerjee, 2004).

Infection of A549 cells by VSV G protein-pseudotyped lentivirus expressing enhanced GFP (lenti-VSV). To study the cellular entry efficiency of VSV, we constructed a VSV G (envelope protein)-pseudotyped lentivirus expressing the enhanced GFP (eGFP) gene (lenti-VSV) (Popik et al., 2002; Yonezawa et al., 2005). The transduction efficiency of lenti-VSV (representing cellular entry via VSV G protein) was tested in IFN- and IFN-CM-treated A549 cells. For these studies, A549 cells were either left untreated or incubated with IFN-α (1000 U ml⁻¹) or conditioned medium (MCM or IFN-CM) for 8 h. The cells were then washed and lenti-VSV (m.o.i. of 20) was added in the absence of IFN or conditioned medium. At 40 h p.i., eGFP expression was determined by fluorescence microscopy.
RESULTS

Rapid establishment of IFN-mediated antiviral activity in A549 cells

Initially, we performed a series of experiments to delineate the kinetics of the antiviral action of IFN. It is well established that pre-treatment of cells with IFN for 12–18 h elicits a potent antiviral activity (in the presence of IFN during infection) (Kuwata et al., 1977; Zhao et al., 1996; Trottier et al., 2005). To investigate whether shorter IFN pre-treatment would result in inhibition of VSV replication, A549 cells pre-treated with IFN for 0–5, 1 and 16 h were infected with VSV in the presence of IFN. At 36 h p.i., viral titre and infection efficiency were determined by plaque assay and Western blot analysis using anti-VSV P antibody. As expected, in cells pre-treated with IFN for 16 h there was a dramatic decline of 4 log in infectivity (Fig. 1a). However, there was also a significant inhibition (100- to 300-fold) of virus infectivity in cells pre-treated with IFN for only 0–5 and 1 h. Concomitantly, Western blot analysis (Fig. 1b) revealed complete abolishment (the titre was decreased by more than 100-fold) of intracellular VSV P protein production following treatment of cells with IFN for 1 h. These results suggested that pre-treatment of cells with IFN for 0–5–1 h may be sufficient to induce a robust antiviral activity.

Next, we repeated the experiments but included cells infected in the absence of IFN. Cells pre-treated with IFN (1 or 18 h) were infected with VSV in the presence or absence of IFN. As expected, a significant decline in infectivity was observed in cells pre-treated for 18 h and infected in the presence of IFN (Fig. 1c). Surprisingly, significant antiviral activity was retained in the absence of IFN during infection. Interestingly, pre-treatment of cells for only 1 h (in the absence of IFN during infection) preserved substantial levels of antiviral activity. These results were confirmed by Western blot analysis (Fig. 1d), as pre-treatment for 1 h (in the absence of IFN during infection) led to complete loss of VSV P protein synthesis. In contrast, IFN failed to inhibit virus replication when it was added following viral infection (4–8 h p.i., in the absence of pre-treatment) (data not shown). These results demonstrated that IFN can bring about an antiviral state within a short period of time (1 h) and that IFN-induced antiviral factors sustain this activity during infection. These results also suggested that IFN may induce an antiviral mechanism/strategy very rapidly, resulting in the production of antiviral factors such as soluble secreted factors, which are known to be produced rapidly following stimulation of cells (Landis et al., 1991; Nevins & Thurmond, 2003; Gao et al., 2004). Therefore, we embarked on studies to investigate whether IFN induced such soluble secreted factors to restrict virus infection.

Fig. 1. Pre-treatment (p.t.) of A549 cells with IFN-α for 1 h induces an antiviral state. (a) Culture supernatants collected from untreated (−) and IFN pre-treated A549 cells infected with VSV were analysed by plaque assay. IFN was present during VSV infection (i.e. post-infection, p.i.) of IFN pre-treated cells. (b) A549 cell lysates (10 μg protein) obtained from mock-infected (lane 1) and VSV-infected (lanes 2 and 3) cells (at 36 h p.i.) in the absence (lane 2) or presence (lane 3; cells were pre-treated for 1 h and IFN was present during infection) of IFN were subjected to Western blot analysis with VSV anti-P antibody. (c) Culture supernatants collected from untreated (−) and IFN pre-treated (1 and 18 h) A549 cells infected with VSV were analysed by plaque assay. The presence (+) or absence (−) of IFN after infection (p.i.) is indicated. (d) A549 cell lysates (10 μg protein) obtained from mock-infected (lane 1) and VSV-infected (lanes 2–6) cells (at 36 h p.i.) in the absence of IFN (lane 4) or following pre-treatment of cells with IFN for 18 h (lanes 2 and 3) or 1 h (lanes 5 and 6) were subjected to Western blot analysis with VSV anti-P antibody. The presence (+) or absence (−) of IFN after infection (p.i.) is indicated.
Antiviral activity of conditioned medium derived from IFN-treated A549 cells

In order to assess the possibility that IFN-induced soluble secreted antiviral factors are produced following IFN treatment, conditioned medium was prepared from IFN-treated cells (Fig. 2a). A549 cells were treated with IFN-α (1000 U ml\(^{-1}\)) for 1–2 h, followed by exhaustive washing to remove exogenous IFN. Fresh medium was added to washed cells, and after 24 h the medium (IFN-CM) was collected. Fresh A549 cells were incubated with IFN-CM for 1 or 8 h, followed by infection of these cells with VSV (in the presence of IFN-CM). Plaque assays (Fig. 2b) of IFN-CM-treated cells demonstrated significant inhibition (2 logs) of virus infectivity compared with untreated and cells treated with the control mock conditioned medium (MCM). The antiviral specificity of IFN-CM was confirmed by the observation that, similar to MCM, IFN-CM prepared from human IFN-α-treated L929 cells (human IFN is not recognized by the mouse IFN receptor) failed to restrict virus infection (data not shown). We utilized 1000 U IFN-α ml\(^{-1}\) to prepare IFN-CM for our subsequent studies, as this represented the optimal concentration required for robust antiviral activity of IFN-CM (based on titration experiments; data not shown). Western blot analysis (Fig. 2c) confirmed the antiviral activity of IFN-CM, as VSV P protein was not detected in cells incubated with IFN-CM. Similar results were obtained when cells were pre-treated with IFN-CM and infected with VSV in the absence of IFN-CM (data not shown), indicating that IFN-CM contains soluble secreted antiviral factors that are induced by IFN-α.

The antiviral activity of IFN-CM is not mediated by IFN-α/β

As IFN-CM was prepared from IFN-α-treated cells, the antiviral activity of IFN-CM could have been due to a residual amount of IFN-α present in the conditioned medium or due to production of de novo-synthesized IFN-α/β. However, these possibilities are unlikely as (i) the residual amount of IFN-α present after exhaustive washing would be endocytosed and rapidly degraded (Zoon et al., 1983); (ii) IFN-α treatment does not induce the IFN-α/β gene (Der et al., 1998); and (iii) although IFN-α and IFN-β are acid-stable cytokines (Nakane & Minagawa, 1981), acid treatment of IFN-CM eliminated its antiviral activity (data not shown). The lack of involvement of IFN-α/β during IFN-CM-mediated antiviral action was confirmed by further assays. Firstly, quantification of IFN-α and IFN-β concentrations in IFN-CM using a highly sensitive ELISA failed to detect IFN-α in IFN-CM, whereas it was able to detect 10 and 100 U recombinant IFN-α added exogenously to regular medium (DMEM) or to the conditioned media derived from A549 cells (Table 1). Similar results were obtained by performing an IFN-β-specific ELISA (data not shown). Secondly, IFN-α-specific (Fig. 3a) and IFN-β-specific (data not shown) neutralizing antibodies failed to alter the antiviral activity of IFN-CM. The neutralizing activity of these antibodies were specific, as they dramatically inhibited the antiviral activity of exogenously added

![Fig. 2. Conditioned medium (IFN-CM) prepared from IFN-α-treated cells inhibits VSV infection.](image)

Table 1. Measurement of IFN-α concentration in conditioned media by ELISA analysis

<table>
<thead>
<tr>
<th>Sample</th>
<th>IFN-α (pg)</th>
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<tr>
<td>10 U IFN-α + DMEM</td>
<td>55 ± 2.8</td>
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<tr>
<td>100 U IFN-α + DMEM</td>
<td>580 ± 17.4</td>
</tr>
<tr>
<td>10 U IFN-α in A549 MCM</td>
<td>51 ± 3.6</td>
</tr>
<tr>
<td>10 U IFN-α in A549 IFN-CM</td>
<td>56 ± 4.1</td>
</tr>
<tr>
<td>10 U IFN-α in Vero MCM</td>
<td>53 ± 3.4</td>
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<tr>
<td>10 U IFN-α in Vero IFN-CM</td>
<td>57 ± 3.9</td>
</tr>
<tr>
<td>A549 MCM</td>
<td>ND</td>
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<tr>
<td>A549 IFN-CM</td>
<td>ND</td>
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<tr>
<td>Vero MCM</td>
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<td>Vero IFN-CM</td>
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Values are means ± SD. ND, Not detected (IFN-α concentration below the detection limit). The minimum detectable amount of IFN-α in the ELISA was 0.1 U.
Following incubation of A549 cells with IFN-CM (data not shown), thus confirming the absence of IFN-α/β. It is important to mention that IFN-CM prepared from monkey CV-1 fibroblast cells (sensitive to human IFN-α/β, similar to Vero cells) failed to restrict VSV infection in both CV-1 and A549 cells (data not shown). Based on these observations, we suggest that IFN-induced soluble factors may constitute epithelial-cell-specific proteins.

Based on these results, it was clear that the antiviral activity of IFN-CM was not elicited by IFN-α/β. We speculated that IFN-induced soluble antiviral factors might restrict infection at the stage of cellular entry of VSV, since (i) soluble secreted factors are extracellular factors, and (ii) VSV infection was restricted in cells pre-treated with IFN-CM (in the absence of IFN-CM during infection). IFN treatment of cells might also lead to inhibition of VSV entry, as pre-treatment of cells with IFN for only 1 h (in the absence of IFN during infection) reduced VSV infectivity (Fig. 1c, d).

**IFN and IFN-CM inhibit cellular entry of VSV**

The efficiency of virus entry in IFN- and IFN-CM-treated cells was next investigated by utilizing two techniques. First, we pre-treated A549 cells with IFN for 8 h and infected them with VSV (m.o.i. of 10) in the presence of cycloheximide (100 μg ml⁻¹). After adsorption for 45 min, cells were washed and fresh medium containing cycloheximide was added in the absence of IFN. Cells were fixed and labelled with anti-VSV P protein antibody and FITC-labelled secondary antibody at 45 min p.i., and the amount of intracellular P protein was detected by immunofluorescence microscopy. By performing infection in the presence of cycloheximide, a protein synthesis inhibitor (Rigaut et al., 1991), this technique enabled us to detect virus entry by monitoring the amount of intracellular P protein derived from the input virus, as the infection time frame is very short (prior to transcription/replication initiation). The results (Fig. 4a) clearly demonstrated dramatic inhibition of VSV entry in the presence of IFN, as the amount of intracellular VSV P protein (from input virus) was significantly reduced in IFN-treated cells. Similarly, IFN-CM treatment also significantly inhibited VSV entry into A549 cells (Fig. 4b).

To confirm these findings, we next studied the ability of IFN and IFN-CM to inhibit the entry of VSV G-pseudotyped lentivirus expressing eGFP (lenti-VSV), as described in Methods. Pseudotyped viruses containing heterologous viral envelope protein have been widely used to study virus entry (Popik et al., 2002; Yonezawa et al., 2005). For our studies, A549 cells were incubated with IFN or IFN-CM for 8 h, followed by the addition of lenti-VSV (m.o.i. of 20). Following adsorption for 4 h, cells were washed and fresh medium was added in the absence of IFN or IFN-CM. At 40 h p.i., the expression of eGFP was monitored by fluorescence microscopy. Pre-treatment of cells with IFN (Fig. 4c) or IFN-CM (Fig. 4d) resulted in a dramatic inhibition of VSV entry, with significant reduction of eGFP expression in these cells compared with untreated cells.

IFN-α and IFN-β (100 and 1000 U ml⁻¹) (Fig. 3a). Note that 10 U IFN-α ml⁻¹ failed to inhibit VSV replication efficiently, confirming the non-involvement of IFNs during the antiviral action of IFN-CM, as the amount of IFN in IFN-CM was significantly lower than 10 U IFN-α (Table 1) or IFN-β (data not shown) ml⁻¹.

Finally, we confirmed the non-involvement of IFN-α/β by utilizing Vero monkey epithelial cells, which are unable to produce IFN-α/β (Zhang et al., 2005). Vero cells are highly sensitive to the antiviral action of human IFN-α (Fig. 3b). Although Vero cells are not capable of producing IFN-α/β, IFN-CM prepared from Vero cells, when added to fresh Vero or A549 cells, inhibited VSV infection by more than 100-fold (Fig. 3b). Moreover, A549 cell-derived IFN-CM was capable of restricting VSV infection following pre-treatment of Vero cells (data not shown). In addition, we failed to detect induction of the IFN-inducible gene PKR following stimulation of Vero cells with IFN-CM (data not shown), thus confirming the absence of IFN-α/β present in IFN-CM.

**Fig. 3.** Antiviral activity of IFN-CM is not mediated by IFN-α/β. (a) Culture supernatants collected from VSV-infected A549 cells either left untreated (UT) or incubated (8 h pre-treatment) with control MCM or IFN-CM (derived from A549 cells) in the presence or absence of IFN-α-neutralizing antibody (α-Ab) were subjected to plaque assay analysis. Similarly, VSV-infected A549 cells pre-treated (16 h) with either α-Ab alone or 1000, 100 or 10 U IFN-α ml⁻¹ in the presence or absence of α-Ab were assayed for infectivity. (b) Plaque assay analysis of VSV-infected Vero cells either left untreated or pre-treated with either IFN-α (1000 U ml⁻¹, 16 h) or with MCM or IFN-CM (8 h pre-treatment) derived from Vero cells. A similar analysis was performed following the addition of Vero cell-derived conditioned medium to A549 cells.

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Together, these results confirmed that, in A549 cells, IFN and IFN-CM treatment lead to inhibition of VSV entry. This demanded further characterization of the soluble secreted factors. Therefore, we performed additional experiments to demonstrate that IFN-induced soluble secreted factors were heat- and acid-sensitive proteins, as the antiviral activity of IFN-CM was lost following heat and acid pH treatment and incubation of IFN-CM with trypsin (Bampton & Taylor, 2005) (data not shown).

**IFN-induced secreted proteins confer antiviral function independently of the STAT1-mediated JAK/STAT pathway**

As the soluble secreted antiviral proteins were produced following IFN treatment and presumably by the subsequent activation of the JAK/STAT pathway, it is possible that IFN-like molecules may constitute secreted antiviral proteins. Moreover, these proteins may activate the JAK/STAT pathway to elicit its antiviral effect. Therefore, we examined the involvement of the JAK/STAT pathway in the antiviral function of IFN-CM. For these studies, we utilized wild-type (2fTGH) and JAK/STAT pathway-deficient (U3A cells lacking STAT1) human fibrosarcoma epithelial cells (Bose _et al._, 2003a; Cremer _et al._, 2002). These cells were incubated with IFN-CM prepared from A549 cells for 8 h and then infected with VSV (in the absence of conditioned medium) for 36 h. Plaque assays (Fig. 5a) revealed the ability of IFN-CM to restrict VSV infection in both wild-type (2fTGH) and STAT1-null (U3A) cells. Moreover, Western blot analysis (Fig. 5b) demonstrated concomitant loss of intracellular VSV P protein in both wild-type and STAT1-null cells. These results suggested that STAT1 and presumably a functional JAK/STAT pathway are not required for the antiviral activity of IFN-CM, although they may be critical for production of soluble secreted antiviral proteins following IFN treatment.
The STAT1-dependent JAK/STAT pathway is not required for the production of secreted antiviral proteins

In order to study the requirement of the STAT1-dependent JAK/STAT pathway for the induction of soluble secreted antiviral proteins, we prepared IFN-CM from wild-type and STAT1-null cells by the procedure described in Fig. 2(a). A549 cells were incubated with these conditioned media for 8 h, followed by virus infection (in the absence of conditioned medium) for 36 h. Surprisingly, we observed that IFN-CM derived from both wild-type and STAT1-null cells retained its antiviral property. As shown in Fig. 6(a), plaque assays demonstrated the ability of IFN-CM generated from wild-type and STAT1-null cells to restrict VSV infection of A549 cells. Moreover, we observed complete loss of intracellular VSV P protein in A549 cells incubated with IFN-CM derived from wild-type and STAT1-null cells as assayed by Western blot analysis (Fig. 6a). In addition, IFN-CM derived from STAT1-null cells restricted VSV infection following its addition to either wild-type or fresh STAT1-null cells (data not shown). ELISA (Table 1) and a neutralization assay (Fig. 3a) revealed that the antiviral activity of IFN-CM derived from wild-type and STAT1-null cells was not due to IFN-α/β. These results indicated that IFN-induced soluble antiviral proteins are secreted into the medium by a STAT1-independent mechanism.

The antiviral properties of IFN are partially retained in STAT1-deficient cells

To study the observed alternative STAT1-independent pathway/strategy, we examined the antiviral efficiency of IFN in wild-type and STAT1-null cells, as this mechanism would be intact in the latter. 2fTGH and U3A cells were pretreated with IFN for 16 h, followed by VSV infection in the presence of IFN as described above for A549 cells. As expected, plaque assays revealed potent antiviral activity of IFN in wild-type 2fTGH cells, with viral titres reduced by almost 6000-fold in these cells (Fig. 7a). In contrast, IFN treatment of STAT1-null U3A cells failed to reduce the viral titre dramatically (Fig. 7a), possibly due to the lack of the JAK/STAT pathway, the major antiviral pathway induced by IFN. In spite of the reduced antiviral properties of IFN in STAT1-null cells, we noted consistently that, in these cells, IFN was still able to reduce the viral titre by approximately 20-fold. A comparison of the fold decrease in viral titre (as determined by plaque assays) following IFN treatment of wild-type and STAT1-null cells is shown in Fig. 7(b).

These results indicated that, although IFN failed to confer a potent antiviral function in STAT1-null cells, partial antiviral activity of IFN was retained in these cells, which...
were devoid of JAK/STAT signalling. Thus, it seems that IFN may utilize an alternative JAK/STAT-independent pathway and/or mechanism to elicit its antiviral action. Based on our results showing that IFN induces soluble secreted proteins independently of the JAK/STAT pathway (Fig. 6), it could be speculated that such a mechanism may be operational during IFN-mediated antiviral action in STAT1-null cells. Indeed, STAT1-null cells were competent in producing soluble antiviral factors following IFN treatment (Fig. 6).

**DISCUSSION**

Our studies to understand the mechanism of antiviral action of IFN stemmed from earlier studies demonstrating that IFN is able to confer antiviral activity against VSV and HPIV-3 independently of three well-established IFN-induced antiviral proteins, PKR, OAS/RNaseL and Mx (Choudhary et al., 2001; Dong et al., 2004; Pattyn et al., 1999; Zhou et al., 1999). The majority of studies focusing on the antiviral activity of these three cytoplasmic proteins were performed using cells that overexpressed these proteins (Staeheili & Pavlovic, 1991; Zhao et al., 1996). As the level of overexpressed proteins does not always reflect the degree of expression of these proteins in IFN-treated cells, it is difficult to assess the antiviral function of these proteins following IFN treatment. Therefore, our initial studies aimed to investigate the mechanism of antiviral function of IFN in context for identification and characterization of novel IFN-induced antiviral factors and the role of the JAK/STAT pathway in IFN-mediated antiviral activity. We observed that IFN conferred significant antiviral activity after pre-treatment of A549 cells for 1 h. The rapid establishment of an antiviral state by IFN led to our identification of IFN-induced soluble secreted factors, which were secreted rapidly (Landis et al., 1991; Nevins & Thurmond, 2003).

Extracellular soluble factors must act early during infection, at a stage when the virus is engaged in the entry process. Indeed, the IFN-induced secreted proteins restricted the entry of VSV into A549 cells. It has generally been considered that, following IFN pre-treatment, VSV attaches to the cell surface and endocytoses normally. The principal restriction in virus replication occurs at the primary transcription step where, presumably, the putative IFN-induced antiviral factors inhibit transcription by the incoming virus particle (Belkowski & Sen, 1987; Dong et al., 2004; Marcus & Sekellick, 1978; Thacore, 1978; Zhang & Samuel, 1987). However, in these studies, VSV entry was monitored by measuring internalization of $[^{35}S]$methionine-labelled virus. Although we and others have widely utilized radiolabelled viruses to monitor entry (Bose & Banerjee, 2002; Bose et al., 2001, 2004), the technique does not differentiate between complete internalization/endocytosis of the virus and incomplete attachment of the virus on the cell surface in a hemi-fusion state (Song et al., 1991). Therefore, erroneous results may arise from using $[^{35}S]$-labelled virus for entry assays, as the cell lysates used for measuring virus-associated radioactivity will include virus present on the cell surface in a hemi-fusion state, a state constituting incomplete ‘mixing’ of the viral envelope lipid bilayer with that of the plasma membrane. To circumvent these problems, we used two techniques to study virus entry: (i) adsorption and penetration of VSV in cells in the presence of cycloheximide, followed by the detection of input viral protein P by immunofluorescence microscopy; and (ii) VSV G-pseudotyped lentivirus expressing the marker eGFP gene. The first technique enabled us to detect virus entry by monitoring the amount of intracellular P protein derived from the input virus, and a similar technique has been utilized previously to study entry/uncoating of VSV (Rigaut et al., 1991). The advantage of the latter technique is that lentivirus is a non-replicating virus and expression of the marker gene (eGFP) serves as a tool to calculate entry efficiency via the VSV G protein used for pseudotyping (Yonezawa et al., 2005). Utilizing these two techniques, we demonstrate that IFN-induced soluble secreted antiviral factors restricted cellular entry of VSV into A549 cells. Interestingly, similar to our results, IFN has been shown to inhibit internalization of VSV into several cell lines (Bukholm et al., 1990).
Subsequently, we observed that IFN-induced soluble antiviral proteins were produced independently of the STAT1-dependent JAK/STAT pathway. Several recent studies have confirmed that, in addition to the JAK/STAT pathway, IFN also induces several other signalling cascades including the PI3 kinase and MAP kinase pathways (Uddin et al., 1997, 2000). At this time, we do not know how these pathways contribute to the antiviral function of IFN. Nevertheless, recent in vivo studies with mice have demonstrated clearly that IFN is able to exert its antiviral activity via a JAK/STAT-independent pathway (Gil et al., 2001). Therefore, we speculate that IFN produces soluble factors very rapidly to restrict virus infection early during infection prior to induction of JAK/STAT-dependent cytoplasmic antiviral proteins such as PKR, OAS and Mx. The critical role of these soluble antiviral factors is also evident from our recent observation that IFN-CM also inhibited HPIV-3 infection of A549 cells (S. Bose, unpublished data).

In summary, our current studies have provided several novel insights into the antiviral mechanisms of IFN including (i) the important role of soluble secreted antiviral proteins (in addition to IFN-induced cytoplasmic proteins) during the IFN-mediated antiviral response; (ii) the inhibition of cellular entry of VSV by the IFN-induced soluble proteins; and (iii) the production of secreted antiviral proteins independently of the JAK/STAT pathway. As IFN activates a wide spectrum of antiviral proteins, we speculate that soluble secreted proteins constitute one arm of the antiviral defence mechanism of IFN. Moreover, rapid activation of these antiviral proteins is advantageous, as these factors could counteract virus infection in the early stages (cellular entry stage). Thus, the co-operative action of multiple antiviral factors (both extracellular and cytoplasmic) is required for complete inhibition of virus infection by IFN. Future studies aimed at characterizing the IFN-induced soluble secreted proteins and investigating the JAK/STAT-independent signalling mechanism in IFN-treated cells will lead to a better understanding of the innate immune antiviral function of IFN.

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