The Zinc Finger Antiviral Protein Acts Synergistically with an Interferon-Induced Factor for Maximal Activity against Alphaviruses

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Type I interferons (IFNs) signal through specific receptors to mediate expression of genes, which together confer a cellular antiviral state. Overexpression of the zinc finger antiviral protein (ZAP) imparts a cellular antiviral state against Retroviridae, Togaviridae, and Filoviridae virus family members. Since ZAP expression is induced by IFN, we utilized Sindbis virus (SINV) to investigate the role of other IFN-induced factors in ZAP’s inhibitory potential. Overexpressed ZAP did not inhibit virion production or SINV-induced cell death in BHK cells deficient in IFN production and thus IFN signaling, suggesting a role for an IFN-induced factor in ZAP’s activity. IFN pretreatment in the presence of ZAP resulted in greater inhibition than IFN alone. Using mouse embryo fibroblast (MEF) cells deficient in Stat1, we showed that signaling through the IFN receptor is necessary for IFN’s enhancement of ZAP activity. Unlike in BHK cells, however, overexpressed ZAP exhibited antiviral activity in the absence of IFN. In wild-type MEFs with an intact Stat1 gene, IFN pretreatment synergized with ZAP to generate a potent antiviral response. Despite failing to inhibit SINV virion production and virus-induced cell death in BHK cells, ZAP inhibited translation of the incoming viral RNA. IFN pretreatment synergized with ZAP to further block protein expression from the incoming viral genome. We further show that silencing of IFN-induced ZAP reduces IFN efficacy. Our findings demonstrate that ZAP can synergize with another IFN-induced factor(s) for maximal antiviral activity and that ZAP’s intrinsic antiviral activity on virion production and cell survival can have cell-type-specific outcomes.

Alphaviruses are positive-sense RNA viruses in the family Togaviridae, whose members cause significant disease to livestock and humans (reviewed in reference 10). Cycling between mosquito vectors and vertebrate hosts, New World members of this genus are responsible for summertime epidemics of equine encephalitis. Human infection can also result in encephalitis for which there is currently no specific therapy. Similarly, summertime epidemics of polyarthritis, fever, and rash can occur upon human infection with Old World alphavirus members.

The alphaviruses share a common replication strategy (reviewed in references 14 and 28) that has been extensively studied in, among other viruses, Sindbis virus (SINV). After virus entry via receptor-mediated endocytosis, fusion of the virion membrane with the endosomal membrane occurs, releasing the nucleocapsid into the cytoplasm. After uncoating of the RNA, the 5’-two-thirds of the SINV genome of ~11,700 nucleotides is translated to generate a polyprotein that is co- and posttranslationally processed to form the nonstructural proteins (nsPs), nsP1, nsP2, nsP3 as well as nsP4, the RNA-dependent RNA polymerase. Together with host-derived factors, the nsPs form a replicase complex, which produces new genomic RNA through a negative-strand intermediate. A subgenomic RNA, also produced from the negative-strand inter-

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terminal 254 amino acids of ZAP fused to the zeocin resistance gene (NZAP-Zeo), exhibits antiviral function in cells of rat (Rat2, fibroblast) as well as human (TREx-293, kidney epithelial) origin (2; also unpublished data). Although not active against a number of viruses, including vesicular stomatitis virus (VSV), yellow fever virus, and herpes simplex virus (2), rat ZAP exhibits antiviral activity against diverse viruses including, in addition to alphaviruses, Moloney murine leukemia virus (MLV) (9) and Ebola virus (23). This suggests that it may have evolved to protect cells against specific viral pathogens. Previous studies demonstrated that endogenous ZAP expression is induced upon stimulation of murine dendritic (25) and human hepatic (20) cells with type I IFNs. Furthermore, infection of primary cells with SINV (25) or human cytomegalovirus (CMV) (5) results in up-regulation of ZAP expression, which is dependent on the type I IFN receptor or IRF3, respectively. Taken together, these suggest that ZAP is an ISG that mediates antiviral activity against viruses from divergent families. Although a number of ISGs have been identified (6), the mechanisms by which the gene products mediate virus inhibition are poorly understood. ISG products likely exhibit cellular activities to which specific viruses display susceptibility. We hypothesized that maximal antiviral activity against SINV might require additional IFN-induced factors and would, therefore, require functional IFN signaling pathways. However, overexpression of rat ZAP is able to mediate significant antiviral activity in the absence of IFN treatment of cells (2).

Recent studies have implicated constitutive, low-level IFN signaling in preparing cells for maximal antiviral responses (reviewed in reference 29). A low level of expression of ISGs might therefore complement the vector-expressed ZAP to mediate maximal antiviral activity. Here, we investigate rat ZAP’s anti-SINV activity in cells defective in IFN production or signaling. Our studies suggest that ZAP inhibition of SINV replication occurs maximally in the presence of another IFN-induced factor(s) and that the effect of ZAP on preventing SINV genome translation renders cells less permissive to virion production in a cell-type-specific manner.

MATERIALS AND METHODS

Cell lines. BHK-21 and Rat2 cell lines transduced with vectors pBabe-HAZ or pBabe-NZAP-Zeo (9) and designated here Rat2/HA-Zeo and Rat2/NZAP-Zeo cells were maintained as previously described (2). The retroviral vector pBabe-pBabe-NZAP-Zeo (9) and designated here Rat2/HA-Zeo and Rat2/NZAP-Zeo was maintained in DMEM containing 10% FBS and 200 g/ml blasticidin.

Production of VSV-G-pseudotyped retroviral particles and cell transductions. MLV particles pseudotyped with VSV protein G (VSV-G) were generated by cotransfection of 293T cells with pBabe-HAZ or pBabe-NZAP-Zeo DNA and DNAs encoding MLV Gag-Pol and VSV-G envelope proteins as described previously (9). Bulk transduced cell lines were obtained by infection of the cells with the pseudotyped viral particles and selection in the presence of 200 g/ml zeocin. Human immunodeficiency virus particles pseudotyped with VSV-G were obtained by cotransfection of 293T cells with shRNA-expressing derivatives of pLenti-3-U6-EC-EF7 (see “Silencing of endogenous murine ZAP,” below) and DNAs encoding human immunodeficiency virus Gag-Pol and VSV-G. Bulk transduced cell lines were obtained by infection with the pseudotyped viral particles and selection in the presence of 5 g/ml blastidicin.

Silencing of endogenous murine ZAP. The pLenti-3-U6-EC-EF7 plasmid provided by Daniel Boden, Aaron Diamond AIDS Research Center, New York, NY was engineered to express an irrelevant shRNA or shRNA targeting murine ZAP. The shRNA expression vector pLenti-3-U6-EC-EF7 is a derivative of vector pLenti6/V5-D-TOPO (Invitrogen) and was generated as follows (D. Boden, personal communication): briefly, the Topo binding sites and the CMV promoter of pLenti6/V5-D-TOPO were removed, and a BamHI/HpaI polynkner was introduced into the lentiviral 3’ terminal repeat. These two restriction sites were used to insert an shRNA expression cassette containing the U6 poly- merase III promoter preceded by the enhancer of the CMV immediate-early promoter. Two restriction sites (EcorV and PsiI) downstream of the U6 promoter allow for the introduction of short hairpin DNA sequences. Two repeated stretches of six thymidine residues following the PsiI site serve as a polymeric III transcription terminator site. The pLenti-3-U6-EC-EF7 defective lentiviral vector also expresses the blasticidin resistance gene. The murine ZAP mRNA sequence (NM_028864) was used to contain the murine ZAP target sites (W. M. Kech Oligonucleotide Synthesis Facility, Yale University) containing inverted repeats of the 3’ untranslated region of the Renilla luciferase gene in the psicHECK-2 vector (Promega). Efficiency of silencing was assessed as recommended by the manufacturer by cotransfection of the murine ZAP-containing psicHECK derivative into 293T cells with the various shRNA-expressing plasmids. The two plasmids showing the lowest normalized Renilla luciferase activity, as well as plasmid pLenti-3’-irrelevant-shRNA (obtained from Thomas von Hahn, Rockefeller University, New York, NY), were utilized to transduce wt MEF/HA-Zeo and wt MEF/HA-Zeo cells to generate constitutively silenced cell lines. Sequences of the sense and antisense oligonucleotides utilized for generation of the two silenced cell lines (wt MEF/HA-Zeo/shRNA-ZAP-1 and wt MEF/HA-Zeo/shRNA-ZAP-5, respectively) were as follows, with the target sequence shown in uppercase letters:

ZAP-1 sense, 5’-atcAAGAGGAAATGCTCATATGTCACATCTCTGACATGTTCCTTCTCTTgat-3’; and ZAP-5 antisense, 5’-gaaaatcGGTCCAGAGTAAGTgatcctACTTACTCTGGACCTCTTCTCTTgat-3’

ZAP-1 luciferase activity, as well as anti-ZAP-1 luciferase activity, in control MEF/HA-Zeo/shRNA-ZAP-1 and wt MEF/HA-Zeo/shRNA-ZAP-5 transfected cells was determined using a Dual-Luciferase assay kit (Promega) according to the manufacturer’s instructions, using random hexamers as primers. cDNAs diluted 1:20 were then amplified with a QuantiTect SYBR Green PCR kit (Qiagen) and detected with a LightCycler 480 (Roche). Enzyme activation occurred at 95° for 15 min, followed by 40 cycles of 94° for 15 s, 55° for 20 s, and 72° for 20 s. Primers for amplification of murine ZAP were obtained from Qiagen (Qiagen primer assay, QT001708). Samples were normalized based on the amount of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) CDNA present, as determined by real-time PCR using Qiagen QuantiTect Primers (QT0039099). Relative levels were determined using samples from duplicate wells, each assayed in triplicate PCRs, using the comparative threshold cycle method (1a). The efficiency of ZAP and GAPDH amplification was tested and found to be approximately equal in the cDNA dilution range utilized in the assay.
**IFN treatment**. Universal type I IFN (PBL Biomedical Laboratories), a recombinant human IFN-α and IFN-β chimeric protein with activity for type I IFN receptors from multiple mammalian species, including hamster and rat, was diluted in medium appropriate for the cell type and utilized for all IFN-α treatments. Duration of treatment is indicated in the figure legends.

**Viruses, infections, and titrations.** Stocks of wt SINV (Toto1101), SINV expressing firefly luciferase as a fusion with nP3 (Toto1101/Luc), a temperature-sensitive mutant SINV expressing luciferase (Toto1101/Luc/tc110), and VSV (San Juan strain) were prepared, and titers were determined on BHK-21 cells as described previously (2). Multiplicity of infection (MOI) was determined based on BHK-21-derived titers. Viral infections, growth curves, and virus titrations were performed as previously described (2). The temperature sensitivity property of the Toto1101/Luc/tc110 virus stock was verified by plaque assay titration on BHK/HA-Zeo cells; no plaques were detected at any dilution (including undiluted stock) at 40°C, while the stock titer, determined at 28°C was >10^7/mL.

**Luciferase assays.** Cell monolayers were washed with phosphate-buffered saline, and lysates were prepared and measured using 1× passive lysis buffer and a luciferase assay system (Promega) according to the manufacturer’s recommendations. Luciferase activity was measured using a Berthold LB960 luminometer.

**Antibody production.** A bacterial expression plasmid (pGXEX-NZAP) producing glutathione-S-transferase (GST) with the amino-terminal 254 residues of rat ZAP (NZAP) fused to the carboxy terminus was generated in plasmid pGXEX-6P-2 (GE Healthcare) using standard techniques. Details of the construction and the sequence of the plasmid are available upon request. Bacterially expressed GST-NZAP was purified on a GSTrap FF column. GST-NZAP and NZAP were utilized as the antigen in the more closely related hamster cells. We tested expression of the NZAP-Zeo protein in the BHK/NZAP-Zeo cells by indirect immunofluorescence (Fig. 2) and Western blotting (see Fig. 5) and found that levels were similar to the level seen in the highly SINV-nonpermissive Rat2/NZAP-Zeo cells (2). Moreover, the diffusely cytoplasmic subcellular distribution (Fig. 2) was similar to that seen in Rat2/NZAP-Zeo cells (not shown).

**Pretreatment with type I IFN restores ZAP’s inhibitory activity in BHK cells.** If, due to defective IFN production (and thus also with reduced constitutive low-level IFN signaling), BHK cells lack an ISG factor necessary for ZAP’s function, then treatment of cells with IFN might restore ZAP’s inhibitory activity in these cells. We pretreated BHK/HA-Zeo and BHK/NZAP-Zeo cells with various doses of IFN-α and assessed the ability of the cells to support SINV growth (Fig. 3A). In BHK cells expressing vector alone, pretreatment with IFN-α had little effect on SINV growth, with only a mild (less than 1 log) reduction in virion production seen at the highest IFN dose tested (100 U/ml) for both low and high MOIs. In contrast, IFN-α pretreatment of BHK/NZAP-Zeo cells resulted in a dose-dependent reduction of SINV growth at both low and high MOIs. Virion production 24 h after infection at a low MOI was reduced by >2 or >4 logs after pretreatment with 10 and 100 U/ml IFN-α, respectively. Similarly, virion production 12 h after infection at a high MOI was reduced by >1 or >2 logs after pretreatment with 10 and 100 U/ml IFN-α, respectively. IFN-α-treated BHK/NZAP-Zeo cells were also protected from SINV-mediated cell death to a greater degree than BHK/HA-Zeo cells (Fig. 3B).

One possible explanation for the SINV inhibition seen in the NZAP-Zeo-expressing cells treated with IFN is that these cells are inherently more responsive to IFN. To test this, we examined the effects of IFN-α pretreatment of BHK/HA-Zeo and BHK/NZAP-Zeo cells on the growth of VSV, a highly IFN-sensitive virus, whose growth is unaffected by ZAP expression.

**Western blotting.** Cells were lysed in 2× Laemml sample buffer, and the proteins in the lystate were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to Hybond ECL (enhanced chemiluminescence) nitrocellulose membranes (GE Healthcare), and incubated with primary and secondary antibodies as previously described (19). Enhanced chemiluminescence detection was performed with SuperSignal West Pico chemiluminescent substrate (Pierce) according to the manufacturer’s recommendation.

**RESULTS**

Rat ZAP fails to inhibit SINV production in cells defective in type I IFN production. BHK-21 cells (hamster kidney) are frequently utilized for the propagation of a variety of viruses, including SINV. The robust and often persistent growth of various viruses is likely due to a deficiency in IFN production by these cells (1, 3, 24, 27, 30), although the deficiency has not been fully characterized, nor has the molecular mechanism(s) of the defect been elucidated. We utilized these cells to test whether rat ZAP is able to mediate its inhibitory effects in cells with a deficiency in IFN production (and thus likely also with little to no IFN signaling). Using VSV-G-pseudotyped MLV vectors pBabe-HAZ and pBabe-NZAP-Zeo (9), we generated stably transduced BHK-21 cell populations expressing the zeocin resistance gene (BHK/HA-Zeo) or NZAP fused to the zeocin resistance gene (BHK/NZAP-Zeo) and examined the growth of SINV. Virus production at both low (Fig. 1A) and high (Fig. 1B) MOIs was found to be virtually indistinguishable in the two cell lines. SINV-mediated cell death was also similar, although a possible slight delay in cytopathic effect (CPE) was noted in the cells expressing NZAP (Fig. 1C).

Possible reasons for the failure of ZAP to inhibit SINV growth and virus-mediated CPE in BHK cells include a species incompatibility, the lack of a necessary factor(s), the presence of a factor(s) that interferes with ZAP’s action, inadequate levels of ZAP expression from the transducing retroviral vector, or an altered subcellular distribution of ZAP in BHK cells. We felt a species incompatibility was unlikely, given the high similarity in ZAP’s amino terminal sequences among different mammalian species (rat NZAP shares 93% and 80% amino acid identity and 94% and 89% similarity with murine and human NZAP, respectively). Additionally, since rat ZAP is able to function in human cells, we felt it was likely to function in the more closely related hamster cells. We tested expression of the NZAP-Zeo protein in the BHK/NZAP-Zeo cells by indirect immunofluorescence (Fig. 2) and Western blotting (see Fig. 5) and found that levels were similar to the level seen in the highly SINV-nonpermissive Rat2/NZAP-Zeo cells (2). Moreover, the diffusely cytoplasmic subcellular distribution (Fig. 2) was similar to that seen in Rat2/NZAP-Zeo cells (not shown).

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The susceptibility of IFN-α/H9251-treated BHK/HA-Zeo and BHK/NZAP-Zeo cells to VSV-mediated CPE was essentially identical (Fig. 4). Over multiple experiments, the BHK/NZAP-Zeo responsiveness to IFN-H11032 effects was always equal to or less than that of the BHK/HA-Zeo cells (not shown). Furthermore, IFN-α pretreatment led to similar reductions in VSV virion production in the two lines, with a slightly reduced effect of IFN-α on VSV growth in the BHK/NZAP-Zeo cells (Table 1). Therefore, the increased ability of IFN-α treatment to inhibit SINV replication in the BHK/NZAP-Zeo cells is not due to an inherent increase in IFN sensitivity.

Another possible explanation for enhanced SINV inhibition in the BHK/NZAP-Zeo cells is that IFN-α treatment could increase NZAP-Zeo expression from the transducing vector and thus potentially increase the inhibitory activity. To test this, we evaluated NZAP-Zeo levels after IFN-α treatment by Western blotting and found that levels were not altered and were similar to the level seen in Rat2/NZAP-Zeo cells (Fig. 5).

Together with the VSV results above, this suggests that the ability of the IFN-α treatment to result in enhanced SINV inhibition in the BHK/NZAP-Zeo cells compared to control cells is due to the expression of NZAP-Zeo, which likely functions in concert with an IFN-induced factor(s).

Enhanced activity of ZAP requires signaling through the type I IFN receptor. If ZAP inhibitory activity requires the presence of an IFN-induced factor, then inhibition of IFN signaling should abrogate the ability of IFN-α to restore ZAP-mediated inhibition of SINV. To examine IFN-α effects on ZAP function in the absence of IFN signaling, we utilized immortalized MEFs generated from mice with a targeted disruption of the Stat1 gene (8) (Stat1−/− mice). Mice deficient in Stat1 have previously been shown to lack responsiveness to IFN-α/β and to be highly susceptible to viral pathogens (8, 22). Using immortalized MEFs from wt mice as well as Stat1−/− mice, we generated cell lines expressing HA-Zeo (wt MEF/HA-Zeo and Stat1−/− MEF/HA-Zeo, respectively) or expressing NZAP-Zeo (wt MEF/NZAP-Zeo and Stat1−/− MEF/NZAP-Zeo, respectively) to examine ZAP’s ability to inhibit SINV in the presence or absence of IFN signaling.

As expected, due to the lack of IFN-α/β responsiveness, IFN

![FIG. 1. ZAP inhibition of SINV replication and virus-induced cell death in BHK cells. (A and B) BHK/HA-Zeo and BHK/NZAP-Zeo cells seeded at 7 × 10⁶ cells per 35-mm dish the day prior were infected with SINV Toto1101 at low (A, 0.01) and high (B, 5) MOIs, and the amount of virus present in the medium was determined at various times after infection by titration on permissive BHK-21 cells. A separate, single well was utilized for each time point; symbols represent the mean of duplicate titrations. (C) Photographs of mock- or SINV-infected BHK/HA-Zeo and BHK/NZAP-Zeo monolayers (MOI of 5) at the indicated times after infection are shown.](http://jvi.asm.org/)

![FIG. 2. Immunofluorescence analysis of NZAP-Zeo expression in BHK/NZAP-Zeo cells. BHK/HA-Zeo and BHK/NZAP-Zeo cells were analyzed by indirect immunofluorescence for the presence of NZAP protein (red) using anti-NZAP monoclonal antibody 13B10.1 and AlexaFluor 594 goat anti-mouse secondary antibody as described in Materials and Methods. The two images shown were acquired with identical exposure conditions under a 60× oil immersion lens. Nuclei are shown in blue.](http://jvi.asm.org/)
pretreatment of Stat1 −/− MEFs expressing either HA-Zeo or NZAP-Zeo had no effect on SINV growth (Fig. 6, top). Surprisingly, however, in contrast to the results obtained in BHK cells defective in IFN production, NZAP-Zeo expression resulted in a SINV growth inhibition despite the inability of these cells to respond to IFN. SINV titers were consistently ~2 logs lower in the NZAP-Zeo-expressing cells for the first 24 h after infection. This suggests that in some cell types, ZAP is able to mediate SINV growth inhibition in the absence of additional IFN-induced factors.

To test whether an IFN-induced factor(s) could further enhance ZAP’s inhibitory activity in these cells, we examined the effect of IFN-α on SINV growth in wild-type (IFN-α/β responsive) MEFs expressing HA-Zeo or NZAP-Zeo (Fig. 6, bottom). The growth of SINV in the wt MEF/HA-Zeo cells, which peaked at ~10⁸ PFU/ml, was reduced compared to the >10⁹

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<th>IFN-α treatment (U/ml)</th>
<th>Virus titer (10⁸ PFU/ml) in the indicated cell line</th>
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<tr>
<td></td>
<td>BHK/HA-Zeo</td>
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<tr>
<td>0</td>
<td>24.1 ± 3.6</td>
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<td>1</td>
<td>20.5 ± 1.8</td>
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a Cells were treated for 11 h with the indicated concentrations of IFN-α and were infected (MOI of 0.01) with VSV. After the cells were washed, IFN-α was added back.

b Virus production was determined after 12 h by titration in duplicate on BHK-21 cells. Results are mean values from two independent wells ± SEM.
PFU/ml seen in the Stat1\(^{-/-}\) MEF/HA-Zeo cells, presumably due to endogenous IFN production and signaling in the wt cells. As expected, pretreatment with IFN-\(\alpha\) resulted in a reduction of SINV growth, with a \(\sim 2\) log reduction seen from 6 to 48 h. In the absence of exogenous IFN treatment, the expression of NZAP-Zeo resulted in a \(\sim 2\) log reduction of SINV growth from 6 to 48 h. IFN-\(\alpha\) pretreatment of wt MEF/NZAP-Zeo cells resulted in enhanced SINV growth inhibition, with a \(\sim 4\) log reduction in titers after 12 h compared to that seen in the untreated wt MEF/HA-Zeo cells. Thus, ZAP and an IFN-induced factor(s) are able to mediate synergistic antiviral effects against SINV.

The synergistic antiviral effect of ZAP and IFN-\(\alpha\) requires new gene transcription. To further support the idea that the effects of IFN-\(\alpha\) on ZAP-mediated SINV inhibition are likely due to production of an ISG factor(s), we examined the effect of transcription inhibition (Fig. 7) on SINV inhibition in wt (IFN responsive) MEFs. Cells expressing HA-Zeo or NZAP-Zeo treated with medium containing vehicle alone (ethanol) showed similar results to the result seen previously (Fig. 6), with a 2-log reduction in viral titers mediated by NZAP expression. Treatment with the cellular transcription inhibitor actinomycin D (ActD) alone had minimal effect on ZAP's inhibitory effect. As expected, treatment of wt MEF/NZAP-Zeo cells with IFN-\(\alpha\) resulted in synergistic inhibition of SINV growth (\(\sim 3\) logs at 24 h compared to that seen in the wt MEF/HA-Zeo cells treated with vehicle alone). The synergistic effect of IFN-\(\alpha\) upon ZAP-mediated SINV inhibition was completely abrogated when IFN-\(\alpha\) treatment was performed in the presence of ActD. The reduced inhibition seen in IFN-\(\alpha\)-treated wt MEF/NZAP-Zeo cells in this experiment (3 logs) compared with the data presented in Fig. 6 (4 logs) is likely due to the short duration of IFN treatment (2 versus 15 h), which was necessary due to toxic effects of ActD (not shown). In the absence of ActD, the effects of IFN-\(\alpha\) were time dependent, with a maximal effect requiring 4 to 6 h of treatment in these cells (not shown). Thus, the synergistic inhibitory effect of IFN-\(\alpha\) with ZAP on SINV takes time and is dependent on new

FIG. 5. Western blot analysis of NZAP-Zeo levels in IFN-\(\alpha\)-treated cells. The indicated cell lines were seeded at \(7 \times 10^5\) cells per 35-mm dish and allowed to adhere. Cells were then treated overnight as indicated with 500 U/ml IFN-\(\alpha\) before harvesting. Equal volumes of lysate were loaded onto 10% polyacrylamide gels and subjected to Western blot analysis using anti-NZAP monoclonal antibody 13B10.1 (top panel). A duplicate blot was probed with anti-\(\beta\)-actin antibodies as a loading control (bottom panel). Similar results were obtained in one other independent experiment.

FIG. 6. ZAP-mediated inhibition in Stat1\(^{-/-}\) and wt MEFs. Stat1\(^{-/-}\) or wt MEFs expressing HA-Zeo or NZAP-Zeo were seeded at \(3 \times 10^5\) (Stat1\(^{-/-}\)) or \(1.2 \times 10^5\) (wt) cells per 35-mm dish and were allowed to adhere and then were incubated overnight (15 h) with normal medium (filled symbols) or medium containing 100 U/ml IFN-\(\alpha\) (open symbols). The cells were then infected with SINV Toto1101 (MOI of 5), and medium without additives was added back. Two separate wells were utilized for each condition, and the medium in each was harvested and replaced at each time point. The amount of virus present in each sample was determined by titration in duplicate on permissive BHK-21 cells, and the cumulative total for each time point is presented. Symbols represent the mean log titers of the duplicate dishes; error bars, often obscured by the symbol, indicate the SEM.

FIG. 7. Effect of transcription inhibition on the ZAP-IFN synergy. wt MEFs expressing HA-Zeo or NZAP-Zeo were seeded at \(1.5 \times 10^5\) cells per 35-mm dish the day prior to treatment for 2 h with medium containing vehicle alone or with medium containing 500 U/ml IFN-\(\alpha\) in the presence or absence of 1 \(\mu\)g/ml ActD. The cells were then washed and infected with SINV Toto1101 (MOI of 5), and medium without additives was added back. Two separate wells were utilized for each condition, and the medium in each was harvested and replaced at each time point. The amount of virus present in each sample was determined by titration in duplicate on permissive BHK-21 cells, and the cumulative total for each time point is presented. Symbols represent the mean log titers of the duplicate dishes; error bars representing the SEM are obscured by the symbols.
In the absence of IFN-α stimulation, similar to the production of ISG factors after IFN-α stimulation.

**IFN-independent ZAP inhibition of SINV replication has cell-type-specific outcomes on virus production.** That ZAP mediates a 2-log SINV reduction in Stat1−/− MEFs incapable of responding to IFN (Fig. 6) suggests that ZAP has intrinsic antiviral activity which functions independently of the activity of other ISGs. In wt MEFs, the synergistic activities of ZAP and an ISG factor have profound effects on virus production. However, in BHK cells ZAP fails to mediate inhibition of SINV growth in the absence of IFN-α treatment. We wondered, therefore, whether the ISG-independent activity was unable to function in the BHK cellular environment or, alternatively, whether the activity was functional but insufficient to result in significant inhibition of virus production.

Previously, using Rat2 fibroblast cells, we found that overexpression of rat NZAP-Zeo dramatically inhibits SINV virion production (~6 logs at 24 h after infection at a low MOI) and found that ZAP mediates its inhibition after entry and at or before translation of the incoming genomic RNA (2). The inhibitory effect did not require pretreatment with IFN. Here, we utilized SINV Toto1101/Luc, which expresses luciferase as an in-frame fusion with the nsP3 protein, and monitored luciferase activity after 2 h of infection in order to investigate ZAP’s effect on early translation and replication events in MEF and BHK cells. In wt MEFs, either expression of NZAP-Zeo or IFN-α pretreatment (100 U/ml) resulted in a >99% reduction in early replication events, as measured by luciferase activity after 2 h of infection (data not shown). This is consistent with the ability of ZAP or IFN-α to significantly reduce virion production, as seen in Fig. 6. Since the luciferase values obtained in the MEFs with NZAP-Zeo expression or IFN-α pretreatment were at background levels, we were unable to assess for synergistic inhibition of the early viral translation and replication events.

Surprisingly, despite a failure to inhibit SINV production in the BHK cellular environment in the absence of IFN (Fig. 1 and 3), expression of NZAP-Zeo was able to significantly decrease early replication events (Fig. 8), even without IFN-α pretreatment. In nine experiments measuring luciferase activity after 2 to 3 h of infection, expression of NZAP-Zeo resulted in a 92% (standard error of the mean [SEM], 1%) reduction in early replication relative to BHK cells expressing HA-Zeo (data not shown). Pretreatment with IFN-α resulted in a dose-dependent decrease in early viral replication in BHK/NZAP-Zeo cells, with higher doses resulting in complete inhibition, similar to the inhibition seen in the highly nonpermissive Rat2/NZAP-Zeo cells, where inhibition is not dependent on exogenous IFN treatment (Fig. 8). While IFN treatment did not significantly alter the levels of early viral replication in Rat2 cells expressing HA-Zeo, a mild reduction was seen in the BHK/HA-Zeo cells. These findings suggest that, in the absence of additional ISGs, ZAP mediates an antiviral effect on early SINV replication events but that the magnitude of the inhibition in some cell types (BHK) is insufficient to alter virus production, while in other cell types (MEFs and Rat2) virion production is decreased. Amplification of the early inhibition, mediated by the synergistic effects of an ISG factor(s), increases the extent of inhibition such that virion production is reduced.

**Like ZAP, the IFN-induced factor(s) targets an early step in the SINV life cycle.** Since SINV Toto1101/Luc is replication competent, luciferase activity would be expected to increase upon translation of any newly replicated viral RNA. Therefore, reductions in luciferase activity with IFN pretreatment (Fig. 8) could be due to inhibition at any one of several steps of the virus life cycle. We previously determined that ZAP mediates its inhibitory step at or prior to translation of the incoming viral RNA (2). To test if the IFN-induced factor(s) was also affecting a similar step, we infected IFN-α-treated cells with a temperature-sensitive derivative of Toto1101/Luc, Toto1101/Luc-ts110 (2), and measured the luciferase activity under nonpermissive conditions where RNA replication is blocked (Fig. 9). An IFN-α dose-dependent reduction of SINV genome
compared to untreated cells. RT-PCR analysis confirmed that treatment of cells expressing the irrelevant shRNA with 6.25 ZAP-specific shRNAs. The level of ZAP RNA induced by untreated cells was apparent in the two cell lines expressing untreated cells, significant replication (set at 100%). Error bars indicated cells were seeded at 2.4 x 10^5 cells per well in 12-well plates the day prior to addition of twofold serial dilutions (50 U/ml to 6.25 U/ml) of IFN-α as indicated. After 6 h, cells were infected with SINV Toto1101/Luc (MOI of 10), after which medium (without IFN-α) was added back. The cells were lysed after 3 h, and firefly luciferase activity was determined. For each cell type, mean luciferase values obtained from mock-infected cells were subtracted from values of infected samples. The bars indicate the relative luciferase activity from IFN-treated cells as a percentage of the mean luciferase values obtained from infected cells without IFN pretreatment (set at 100%).

FIG. 10. Effect of ZAP silencing on antiviral activity of IFN. The indicated cells were seeded at 2.4 x 10^5 cells per well in 12-well plates the day prior to addition of twofold serial dilutions (50 U/ml to 6.25 U/ml) of IFN-α as indicated. After 6 h, cells were infected with SINV Toto1101/Luc (MOI of 10), after which medium (without IFN-α) was added back. The cells were lysed after 3 h, and firefly luciferase activity was determined. For each cell type, mean luciferase values obtained from mock-infected cells were subtracted from values of infected samples. The bars indicate the relative luciferase activity from IFN-treated cells as a percentage of the mean luciferase values obtained from infected cells without IFN pretreatment (set at 100%). Error bars indicated cells were seeded at 2.4 x 10^5 cells per well in 12-well plates the day prior to addition of twofold serial dilutions (50 U/ml to 6.25 U/ml) of IFN-α as indicated. After 6 h, cells were infected with SINV Toto1101/Luc (MOI of 10), after which medium (without IFN-α) was added back. The cells were lysed after 3 h, and firefly luciferase activity was determined. For each cell type, mean luciferase values obtained from mock-infected cells were subtracted from values of infected samples. The bars indicate the relative luciferase activity from IFN-treated cells as a percentage of the mean luciferase values obtained from infected cells without IFN pretreatment (set at 100%).

translation was apparent in BHK cells expressing NZAP-Zeo, suggesting that the IFN-induced factor(s) is also inhibiting early events in the SINV life cycle. Since RNA replication and subsequent virus life cycle steps are blocked at the nonpermissive temperature, these results demonstrate that the IFN-induced factor(s) and ZAP both act at or before translation of the incoming viral RNA genome. Additional IFN-induced blocks at later life cycle steps might contribute to the overall inhibition seen in ZAP-expressing cells infected with replication-competent SINV.

Silencing of endogenous ZAP reduces the antiviral efficacy of IFN-α. While our studies utilizing overexpressed ZAP demonstrated anti-SINV activity, we wondered if ZAP induced by IFN-α treatment might contribute to the antiviral state of the cell. To examine ZAP’s role in the effects of IFN-α, we generated derivatives of wt MEF/Ha-Zeo cells that stably express ZAP-specific shRNAs (wt MEF/Ha-Zeo/shRNA-ZAP-1 or -5) or an irrelevant (wt MEF/Ha-Zeo/shRNA-irrel) shRNA. Treatment of wt MEF/Ha-Zeo/shRNA-irrel cells with IFN-α (100 U/ml for 6 h) resulted in a 4.7-fold increase in ZAP RNA levels (range, 4.6 to 4.8 in duplicate samples), consistent with previous work showing that ZAP is an ISG (20, 25, 31). Treatment of cells expressing ZAP-specific shRNAs with various doses of IFN-α revealed a decreased IFN-mediated antiviral efficacy (Fig. 10). While treatment with a low dose (6.25 U/ml) of IFN-α reduced SINV Toto1101/Luc replication in cells expressing the irrelevant shRNA to less than 5% of that seen in untreated cells, significant replication (~30% of that seen in untreated cells) was apparent in the two cell lines expressing ZAP-specific shRNAs. The level of ZAP RNA induced by treatment of cells expressing the irrelevant shRNA with 6.25 U/ml IFN-α was assessed by quantitative RT-PCR and showed a ~1.7-fold induction (range, 1.67 to 1.76 in duplicate samples) compared to untreated cells. RT-PCR analysis confirmed that silencing of ZAP in the ZAP-1 and ZAP-5 shRNA-expressing cells was efficient. Compared to untreated wt MEF/Ha-Zeo/shRNA-irrel cells, the levels of ZAP RNA in IFN-treated wt MEF/Ha-Zeo/shRNA-ZAP-1 and MEF/Ha-Zeo/shRNA-ZAP-5 cells were reduced, with relative levels of 0.8 (range, 0.71 to 0.84) and 0.2 (range, 0.22 to 0.23), respectively. Even at higher doses of IFN-α, the effect of ZAP silencing was apparent, with higher levels of viral replication occurring in the cells silenced for ZAP expression than in cells expressing the irrelevant shRNA after treatment with 12.5 or 25 U/ml IFN-α (Fig. 10). Treatment with 50 U/ml resulted in minimal virus replication in all three lines, likely due to the induction of multiple ISGs with antiviral activity. That silencing of endogenous ZAP reduces the anti-SINV efficacy of IFN treatment was also recently reported by others (31).

DISCUSSION

Cellular responses to viral infection include activation of signaling pathways, which ultimately lead to the production of type 1 IFNs. IFNs have long been known to confer cellular resistance to viral infection through the induction of antiviral proteins such as the RNA-dependent protein kinase, 2’5’-oligoadenylate synthetase, RNase L, and the Mx family proteins (reviewed in reference 26). IFN treatment of cells, however, induces the expression of a large number of genes (6, 7) whose individual contributions to the cellular antiviral response are poorly understood. While for some proteins, such as human MxA, an intrinsic antiviral effect in the absence of IFN-induced ISG expression has been demonstrated (13), for most ISGs this has not been investigated. ISG20, ISG56, ISG15, ZAP, and viperin genes have been recently implicated in mediating IFN-α’s anti-SINV effects (31). Elucidating whether individual factors exhibit intrinsic antiviral activity or require coexpressed factors is necessary for a full understanding of the IFN-mediated antiviral state. The present study underscores some of the complexities associated with the understanding of the IFN response and in deciphering antiviral mechanisms.

This study focused on the anti-alphaviral activity of ZAP, which, when overexpressed in rat and human fibroblast cell lines, potently inhibits SINV by blocking viral gene expression from the incoming genomic RNA. Similar to findings recently reported (31), we confirmed in this study that ZAP is induced by IFN-α treatment and contributes to the overall antiviral state of the cell (Fig. 10). Since ZAP is induced by IFN, we considered the possibility that the antiviral effect might require the activity of additional IFN-induced factors. Our findings clearly demonstrate that maximal ZAP antiviral activity against SINV occurs in the context of an intact IFN pathway. Overexpression of ZAP fails to inhibit SINV virion production and fails to protect from virus-induced cellular death in BHK cells defective in IFN production (Fig. 1); this failure is overcome by pretreatment of the cells with IFN (Fig. 3) and is not due to low levels of ZAP expression (Fig. 2 and 5). The synergism between IFN and ZAP is not due to a general enhancement of IFN action by ZAP (Fig. 4 and Table 1). Based on this one might conclude that ZAP requires coexpression of an additional IFN-induced factor(s) for its antiviral effect. Surprisingly, however, ZAP exhibits antiviral activity in MEFs derived
from mice with a targeted disruption of the Stat1 gene and thus lacking Stat1-dependent IFN-induced ISG expression (Fig. 6). ISGs induced in a Stat1-independent manner, for example, through signaling mediated by Stat2 or Stat3, are also not likely required for ZAP’s inhibitory effect; no additional antiviral effect was observed when the Stat1−/− MEFs were pretreated with IFN.

The finding of ZAP antiviral activity in some cell types (MEFs) but not apparently in others (BHK) could be due to differences in the intracellular environments of those cell types, for example, in the levels of constitutively expressed proteins. These might be host proteins typically thought of as antiviral (such as many of the ISGs) but also might be factors involved in ongoing cellular processes which the virus subverts or with which it interacts to mediate its life cycle. In addition, however, the amount of antiviral activity attributed to an antiviral factor could depend on the assay utilized to measure antiviral activity. Indeed, we surprisingly found that ZAP exhibits antiviral activity in BHK cells if early gene expression rather than virion production is assessed (Fig. 8 and 9). That this ~1-log decrease in gene expression fails to impact the production of extracellular virus suggests that in BHK cells this step (translation of the incoming viral RNA) is not rate-limiting to SINV production. Pretreatment with IFN, however, results in increased inhibition of early gene expression to levels sufficient to impact virus production and cell survival (Fig. 3 and 8). Furthermore, through the use of a replication-defective SINV mutant, we showed that the IFN-induced factor is synergizing with ZAP’s inhibition of this life cycle step (Fig. 9). Thus, the ultimate outcome of cellular infection with SINV, and likely other viruses, is affected by the constellation of host cellular proteins present, which influence not only cellular antiviral pathways but also the robustness of cellular processes required for each step of the virus life cycle.

In this study we determined that an IFN-induced factor or factors are able to synergize with ZAP to mediate cell-type-specific viral inhibition. While the factor(s) is able to limit a SINV life cycle step at or before that targeted by ZAP, the nature of the factor is unknown. Given that several attempts using a library screening approach have so far been unsuccessful at identifying the responsible ISG product (data not shown), it is possible that IFN treatment reduces or eliminates expression of a factor inhibitory to ZAP’s activity. Alternatively, multiple genes might work in concert to synergize with ZAP. Recently, the IFN-stimulated gene ISG15 was demonstrated to exhibit antiviral effects against several viruses, including SINV (16, 17, 31). IFN treatment is known to induce protein ISGylation (reviewed in reference 15) where, similar to the ubiquitin system, ISG15 protein is conjugated to a variety of proteins. Since in addition to ISG15, IFN-induced activation and conjugation enzymes are necessary for the ISGylation process, multiple gene products would be necessary if IFN’s synergistic effect with ZAP involved ISGylation. Although ZAP has been identified as a possible target for ISGylation in one study (32), IFN treatment of BHK cells does not alter the mobility of ZAP in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 5), and anti-ISG15 Western blot analysis did not detect any bands specific to the IFN-treated BHK/ NZAP-Zeo cells (not shown). Whether ISGylation of ZAP or other cellular proteins plays a role in IFN’s enhancement of ZAP antiviral activity requires further study.

Our study utilized SINV to investigate the role of additional ISGs in ZAP’s inhibitory effect. Since members of the Alphavirus genus share common genomic organization and replication strategies, it is possible that ISGs important for maximal ZAP-mediated anti-SINV activity will also play a role in ZAP’s inhibition of other members of this genus. Although not active against all viruses (2), ZAP also exhibits inhibitory activity towards MLV (9) and both Marburg and Ebola viruses (23). Since these viruses have distinct genomic organizations and replication strategies, it is likely that the cellular requirements for the maximal ZAP-mediated inhibitory effect will be unique to each virus. In fact, we found no evidence for any synergistic effect of IFN pretreatment of BHK cells on ZAP’s inhibitory effect against a luciferase-expressing MLV (not shown). This suggests that ZAP’s inhibitory mechanisms may be both cell type and virus specific.

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