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ISG20, a New Interferon-induced RNase Specific for Single-stranded RNA, Defines an Alternative Antiviral Pathway against RNA Genomic Viruses*

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Interferons (IFNs) encode a family of secreted proteins that provide the front-line defense against viral infections. Their diverse biological actions are thought to be mediated by the products of specific but usually overlapping sets of cellular genes induced in the target cells. We have recently isolated a new human IFN-induced gene that we have termed *ISG20*, which codes for a 3′ to 5′ exonuclease with specificity for single-stranded RNA and, to a lesser extent, for DNA. In this report, we demonstrate that ISG20 is involved in the antiviral functions of IFN. In the absence of IFN treatment, ISG20-overexpressing HeLa cells showed resistance to infections by vesicular stomatitis virus (VSV), influenza virus, and encephalomyocarditis virus (three RNA genomic viruses) but not to the DNA genomic adenovirus. ISG20 specifically interfered with VSV mRNA synthesis and protein production while leaving the expression of cellular control genes unaffected. No antiviral effect was observed in cells overexpressing a mutated ISG20 protein defective in exonuclease activity, demonstrating that the antiviral effects were due to the exonuclease activity of ISG20. In addition, the inactive mutant ISG20 protein, which is able to inhibit ISG20 exonuclease activity *in vitro*, significantly reduced the ability of IFN to block VSV development. Taken together, these data suggested that the antiviral activity of IFN against VSV is partly mediated by ISG20. We thus show that, besides RNase L, ISG20 has an antiviral activity, supporting the idea that it might represent a novel antiviral pathway in the mechanism of IFN action.

Interferons (IFNs)[1] are a family of multifunctional secreted proteins characterized by their abilities to interfere with virus infection and replication (1, 2). IFNs can indirectly inhibit viral production by reducing the growth of target cells and by stimulating their susceptibility to apoptotic processes (3, 4) or by promoting the recognition and the cytotoxic killing of infected cells by the immune system (5, 6). IFNs also act directly at various steps of the viral multiplication cycle through the products of specific but usually overlapping sets of cellular genes induced in the target cells and involved in RNA and protein metabolism and signaling as well (7, 8). Until now, three IFN-regulated pathways have been considered to be involved in these processes: the double-stranded RNA-dependent protein kinase R (PKR) (9–11), the 2-5A/RNase L system (12, 13), and the Mx proteins (14–16). PKR is a serine/threonine kinase that, after binding to dsRNA, phosphorylates the protein synthesis initiation factor eIF2 and the inhibitor of nuclear factor κB (IκB), resulting in the inhibition of protein synthesis and specific transcription regulation (reviewed in Refs. 17–19). RNase L is a dormant cytosolic endoribonuclease that is activated by short oligoadenylates produced by the 2′-5′ oligoadenylate synthetase after viral infection or IFN exposure (reviewed in Refs. 2 and 13). Degradation of viral RNAs and cleavage of cellular 18 S and 28 S rRNAs by the activated RNase L lead to the inhibition of protein synthesis, thus preventing viral propagation (2). Mx proteins are IFN-induced GTPases that interfere with the replication of some negative-stranded RNA viruses by perturbing the intracellular movement and functions of viral proteins (reviewed in Refs. 14 and 15). However, there are now clear evidences for the existence of alternative antiviral pathways beyond the PKR, 2-5A/RNase L, and Mx systems. These evidences were obtained by analysis of mice deficient in all three pathways. Triply deficient mice died 3–4 days earlier than wild type mice after encephalomyocarditis virus (EMCV) infection. However there was still an IFN dose-dependent increase in survival time after viral infection for both wild type and triply deficient mice (20). Moreover, cultured embryonic fibroblasts lacking RNase L, PKR, or both proteins still mounted a substantial residual IFN antiviral response against RNA viruses EMCV or vesicular stomatitis virus (VSV), suggesting that another presently unknown mechanism contributes to the IFN-dependent antiviral response (20).

We have isolated a human cDNA encoding an IFN-induced protein, which we have termed ISG20 for IFN-stimulated gene product of M_r 20,000 (21, 22). ISG20 is a member of the 3′ to 5′ exonuclease superfamily that includes RNases (such as RNase T and D), the proofreading domains of the polymerase I family of DNA polymerases, and DNases that exist as independent proteins (23). Homology within the superfamily is concentrated at three conserved exonuclease motifs termed ExoI, ExoII, and

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[1] The abbreviations used are: IFN, interferon; HuIFN, human interferon; VSV, vesicular stomatitis virus; EMCV, encephalomyocarditis virus; PKR, protein kinase R; dsRNA, double-stranded RNA; wt, wild type; FBS, fetal bovine serum; GFP, green fluorescent protein; MEF, mouse embryonic fibroblast; MOI, multiplicity of infection.

ExoIII (23). Based on these observations, we have demonstrated that ISG20 is a 3′ to 5′ exonuclease *in vitro* with specificity for single-stranded RNA and, to a lesser extent, for DNA, suggesting that ISG20 could be involved in the antiviral function of IFN against RNA viruses (24). Notably, ISG20 is the second known RNase regulated by IFN, along with RNase L (24).

In this report, we have analyzed the ability of ISG20 to protect cells against various viral infections. We demonstrate that in the absence of IFN treatment, ISG20-overexpressing cells showed resistance to infections by VSV, influenza virus, and EMCV but not to adenovirus. A single amino acid substitution in the conserved exonuclease motif ExoII completely abolished both the exonuclease and the antiviral activities of ISG20, demonstrating that the protective effects were due to ISG20 exonuclease activity. We showed that the antiviral action of IFN against VSV was reduced in cells expressing the inactive ExoII mutant of ISG20 protein, suggesting that the antiviral activity of IFN against VSV is partly mediated by ISG20. Because ISG20 is a new RNase induced by IFN, these data support the idea that it might represent a novel antiviral pathway in the mechanism of IFN action.

EXPERIMENTAL PROCEDURES

Cell Cultures—Human HeLa cells and mouse L929 cells were cultured at 37 °C in Dulbecco's modified Eagle's medium supplemented with 10% FBS. HeLa cells overexpressing ISG20 (wt-ISG20) and the inactive mutant of ISG20 (mut-ISG20) were cultured in the same medium supplemented with 1 mg/ml Geneticin (G418; Invitrogen). Mouse embryonic fibroblasts (MEFs) triply deficient in PKR, RNase L, and Mx gene expression were obtained from Dr. R. H. Silverman and cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS. HuIFN-α2a (intron A) was purchased from Schering-Plough. Mouse type I IFN was obtained from Dr. G. Uze.

Construction of ISG20 Expression Vectors—The full-length *ISG20* cDNA fragment was inserted into the pCiNeo expression vector (Promega) to generate the pCiNeo-ISG20 expression construct. The cDNA encoding the inactive ISG20 mutant protein was generated by site-specific mutation in the conserved ExoII motif (24) and subsequently cloned into the pCiNeo vector to generate the pCiNeo-mut-ISG20 expression vector. For the transient transfection experiments, the cDNAs coding for wt-ISG20 and mut-ISG20 protein were subcloned into the pEGFP vector in the same open reading frame as the GFP to generate the pGFP-wt-ISG20 and pGFP-mut-ISG20 expression vector, respectively.

ISG20-expressing HeLa Cells—HeLa cells were transfected by the calcium phosphate procedure with either the pCiNeo-ISG20 or the pCiNeo-mut-ISG20 expression vector. Geneticin (G418; 1 mg/ml) was added to the culture medium 72 h later, and resistant clones were recovered after an additional 2 weeks of selection. ISG20 and mut-ISG20 protein expressions were tested by Western blot analysis with an ISG20-specific mouse polyclonal antibody.

Virus Stocks and Virus Yield Assays—Stocks of VSV (Indiana strain) and EMCV were prepared from supernatants of virus-infected L929 cells. Influenza virus was obtained from Dr. M. Chelbi-Alix. The derivative adenovirus strain bearing a β-galactosidase-encoding reporter gene (β-gal-adenovirus) was obtained from V. Millet. Typically, 5×10^5 cells were plated on 6-well plates and infected for 24 h at 37 °C, in Dulbecco's modified Eagle's medium supplemented with 10% FBS, with VSV, influenza virus, or EMCV at a multiplicity of infection (MOI) of 1 or 0.1. Cell cultures were then frozen and thawed three times. The supernatants were serially diluted, and the virus titers were measured alternatively by a plaque assay on L929 cells as described previously (25) or by an end point method (26). Transient transfection experiments were performed in 6-well plates by the LipofectAMINE Plus Reagent method (Invitrogen). 24 h after transfection, cells were washed twice in phosphate-buffered saline and infected with VSV at an MOI of 0.1 in Dulbecco's modified Eagle's medium supplemented with 10% FBS. Infection with adenovirus was performed in 6-well plates at 10 MOI in RPMI 1640 medium supplemented with 2% FBS. After 2 h, the cells were washed in phosphate-buffered saline, placed in RPMI 1640 medium supplemented with 10% FBS, and then cultured for 24 h at 37 °C before the β-galactosidase assay using the β-Galactosidase Enzyme Assay System (Promega).

Antibodies—Specific ISG20 antibody was developed from the ISG20-glutathione *S*-transferase fusion protein produced in the BL21-DE3 *Escherichia coli* strain and purified by affinity chromatography on glutathione-Sepharose. This material was injected into mice to raise a polyclonal antiserum. The polyclonal ISG20 antibody was used in Western blot analysis to detect endogenous ISG20 protein. Rabbit polyclonal anti-VSV antibodies were described previously (27). Monoclonal antibody against α-tubulin (clone B-5-1-2) was purchased from Sigma Aldrich.

Immunofluorescence Analysis—Confocal immunofluorescence was performed with rabbit polyclonal anti-VSV antibody (27). The cells were fixed for 5 min in phosphate-buffered saline containing 3.7% formaldehyde. VSV antigens were detected with a rabbit anti-VSV antibody (1:500 dilution) and revealed with a fluorescein isothiocyanate-conjugated secondary antibody (Beckman Coulter, Marseille, France). Slides were viewed using a Leika microscopic, and image files were processed with the Adobe Photoshop program.

Western Blotting Analysis—Cells (1×10^6) were resuspended in 50 μl of loading buffer (10 mM Tris-HCl, pH 6.8, 1% SDS, 5 mM EDTA, and 50% glycerol) and incubated for 5 min at 95 °C. The proteins were fractionated on 10% SDS-PAGE and transferred onto a nitrocellulose membrane. After a blocking step, the membrane was hybridized with the appropriate antibodies and then revealed by using a chemiluminescent detection system (ECL; Amersham Biosciences).

RNA Preparation and Northern Blot Analysis—Total RNA was extracted by lysis in guanidinium isothiocyanate as described previously (28). RNA aliquots (20 μg) were electrophoresed through 1.5% agarose/10% formaldehyde gel, transferred onto a nylon membrane (Hybond N^+; Amersham Biosciences), and hybridized to 10^7 cpm/ml ^{32}P-labeled cDNA probe prepared by random priming (Invitrogen). Membranes were washed to a final stringency of 0.2× SSC/0.1% SDS (1× SSC = 0.15 M sodium chloride and 0.015 M sodium citrate, pH 7.0) at 65 °C before autoradiography. The cDNA probes encoding the N, NS, M, and G protein of VSV were obtained from Dr. D. Blondel (25). Hybridization to a glyceraldehyde-3-phoshate dehydrogenase probe (28) was used as an invariant control.

RESULTS

ISG20 Confers Resistance to VSV Infection in Transiently Transfected HeLa Cells—We have shown previously that ISG20 is a 3′ to 5′ exonuclease that specifically degrades *in vitro* single-stranded RNA substrates (and, to a lesser extent, single-stranded DNA substrates) (24). Homology within the superfamily is concentrated at three conserved exonuclease motifs termed ExoI, ExoII, and ExoIII (23). In accordance with this information, a site-specific aspartate to glycine residue mutation in the ExoII conserved motif of ISG20 (mut-ISG20D94G; Fig. 1*A*) abolished its activity (24). Because IFN causes a strong induction of ISG20 expression (21, 29), these data suggested that the protein could be an active player in the antiviral action mediated by IFNs against RNA viruses. The potential antiviral activity of ISG20 against the VSV, a negative-sense RNA genomic virus (rhabdovirus), was evaluated by transient transfection experiments in HeLa cells. The cDNAs coding for both the wild type ISG20 protein (wt-ISG20) and the inactive ExoII-mutated ISG20 protein (mut-ISG20) were cloned under the transcriptional dependence of the cytomegalovirus promoter in the pCiNeo vector. An empty pCiNeo vector was used as a negative control. The cells were transfected with 0.5 or 1 μg of each plasmid and then infected 24 h later with VSV at an MOI of 0.1. 24 h later, the productions of infectious viral particles were determined as described under "Experimental Procedures." The results of a typical experiment, presented in Fig. 1*B*, show that the cells transfected with the wild type ISG20-expressing construct exhibited a dose-dependent reduction in virus production (54.2% for 0.5 μg of transfected DNA and 69.4% for 1 μg of transfected DNA), as compared with cells transfected with the empty pCiNeo vector. Similar results were obtained in three independent experiments. No protective effect was observed in HeLa cells transfected with mut-ISG20, confirming the specificity of the ISG20 antiviral activity. The virus yields were significantly higher in these cells as com-

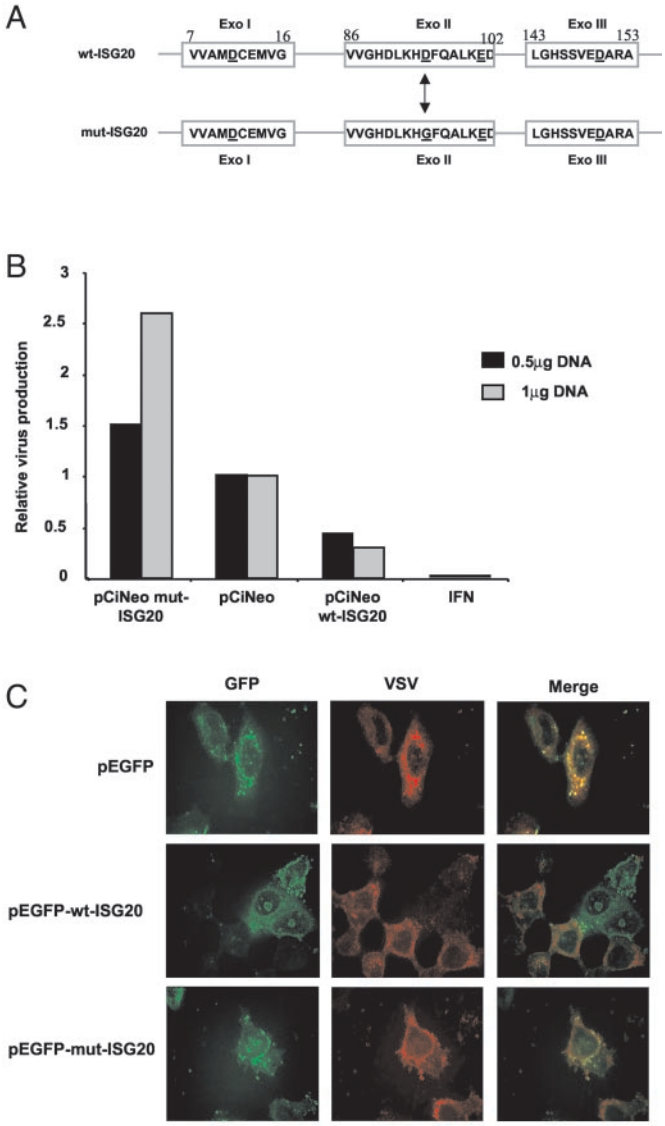

FIG. 1. **ISG20 expression confers resistance to VSV infection in transiently transfected HeLa cells.** *A*, schematic representation of wt-ISG20 and mut-ISG20 proteins. The three exonuclease motifs are indicated, and conserved amino acids are *underlined*. Amino acid substitution is indicated by an *arrow* in position 94. *B*, the cDNAs coding for wt-ISG20 and for the inactive ExoII-mutated ISG20 protein were cloned under the transcriptional dependence of the cytomegalovirus promoter in the pCiNeo vector (pCiNeo-wt-ISG20 and pCiNeo mut-ISG20, respectively). The empty pCiNeo vector was used as a negative control. The cells were transfected with 0.5 μg or 1 μg of each plasmid by the LipofectAMINE Plus Reagent procedure. 24 h after transfections, the cells were infected with 0.1 MOI of VSV. Viral productions were determined 24 h later as described under "Experimental Procedures." The result of a typical experiment is presented. For each experiment, the histogram represents the relative values of viral production calculated by dividing the number of viral particles obtained after transfections with the indicated constructions by the number of viral particles obtained after transfection with the empty pCiNeo vector. For comparison, the relative virus production obtained after treatment of parental HeLa cells with 500 units/ml HuIFN-α2a is presented. *C*, the cDNAs coding for wt-ISG20 and for the inactive ExoII-mutated ISG20 protein were cloned in the pEGFP vector to generate wt-ISG20 (*pEGFP-wt-ISG20*)- and mut-ISG20 (*pEGFP-mut-ISG20*)-fused proteins, respectively. Cells transfected with a vector expressing GFP alone (*pEGFP*) were used as control. HeLa cells were transfected with each plasmid and then infected 24 h later with VSV at 1 MOI. 16 h later, transfected cells were detected by green fluorescence (*GFP*), and VSV antigen expression was monitored by immunofluorescence using a rabbit polyclonal anti-VSV antibody visualized with Texas red (*VSV*). The single confocal image were superimposed (*Merge*).

pared with the cells transfected with the empty vector, suggesting that mut-ISG20 protein might exhibit dominant-negative effects *in vivo* by inhibiting the basal activity of ISG20. These results also suggest that the antiviral action of ISG20 is mediated by its exonuclease activity.

Because, in transient transfection experiments, only a part of the cell population is transfected and expresses the transgene, the antiviral effect observed is underestimated. To circumvent this problem, the anti-VSV ISG20 activity was determined cell by cell by confocal immunofluorescence microscopy. To this end, the cDNAs coding for wt-ISG20 and mut-ISG20 protein were subcloned into the pEGFP vector in the same open reading frame as the GFP to generate the pGFP-wt-ISG20 and pGFP-mut-ISG20 expression vectors. The empty pEGFP vector expressing GFP protein alone was used as a negative control. HeLa cells were transfected with each plasmid and then infected 24 h later with VSV at 1 MOI. 16 h later, transfected cells were detected by green fluorescence, and VSV antigen expression was monitored by immunofluorescence using a rabbit polyclonal anti-VSV antibody described previously (27). As shown in Fig. 1*C*, when HeLa cells were transfected with the pGFP-wt-ISG20 plasmid, the GFP-positive cells that expressed the GFP-wt-ISG20-fused protein did not express detectable VSV antigens, whereas GFP-negative cells exhibited a high level of VSV antigens. At the opposite, all cells expressing either the GFP alone or the GFP-mut-ISG20-fused protein expressed VSV antigens. These data clearly demonstrated the VSV antiviral activity of ISG20.

Constitutive Expression of ISG20 Protein Confers Resistance to VSV Infections—To further analyze the mechanism of ISG20 antiviral action, stable HeLa cells constitutively overexpressing the wt-ISG20 or the mut-ISG20 proteins were constructed (see "Experimental Procedures"). First, mouse specific polyclonal antibodies directed against recombinant ISG20 protein were developed to characterize cells overexpressing ISG20 (see "Experimental Procedures"). According to the modulation of *ISG20* mRNA after IFN treatment (21), the 20-kDa protein detected by the antibodies was induced by IFN in HeLa cells (Fig. 2*A*). These antibodies were used to analyze ISG20 protein expression from stable clones. After Geneticin selection of HeLa cells transfected with either wt-ISG20 or mut-ISG20 constructs, clones expressing wild type ISG20 protein (wt-ISG20) or the inactive ISG20-mutated protein (mut-ISG20) were selected. The data for a representative clone of each population are presented in Fig. 2*A*. ISG20 appears to be expressed at a 3.5 times higher level in wt-ISG20 than in HeLa cells transfected with the empty pCiNeo vector, as determined by densitometry analysis using National Institutes of Health Image software for signal quantification. A higher level of expression was obtained with mut-ISG20 protein, suggesting that the overexpression of wild type ISG20 protein could be critical for cell survival. Because cell proliferation could influence viral infection, we wished to exclude the possibility that variations in virus yields were the result of differences in the growth rate of selected clones. The growth curves established for each clone were similar, excluding this possibility (data not shown).

The selected clones were then infected with 0.1 MOI of VSV. A stable population of HeLa cells transfected with the empty pCiNeo vector (HeLa/pCiNeo) was used to determine viral infection efficiencies. 24 h later, the viral productions were determined as described previously. The mean value of three independent experiments presented in Fig. 2*B* shows a reproducible protective effect, ranging from a 2- to 3-log reduction of viral production in the wt-ISG20 HeLa cells, as compared with the HeLa/pCiNeo cells. A low ISG20 protein expression was

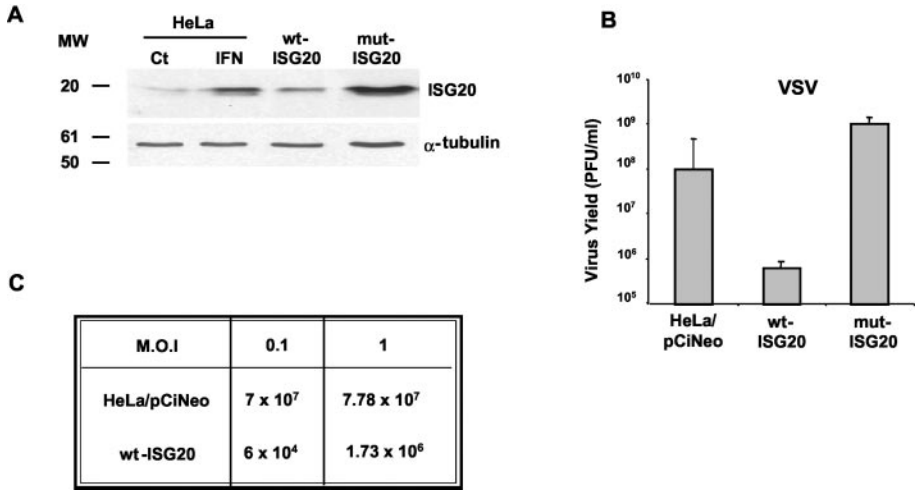

FIG. 2. **Constitutive expression of ISG20 protein confers resistance to VSV infections.** *A*, Western blot analysis of ISG20 protein expression in constitutively wt-ISG20- and mut-ISG20- expressing HeLa clones. Total protein extracts from unstimulated (*Ct*) or stimulated (*IFN*) HeLa cells by 500 units/ml HuIFN-α2a and from constitutively wt-ISG20- or mut-ISG20-expressing clones were analyzed by immunoblotting with a specific mouse polyclonal antibody directed against ISG20 recombinant protein. The sizes of the molecular weight marker (*MW*) and ISG20 protein are indicated. Expression of α-tubulin was used as an invariant control. *B*, the wt-ISG20 and mut-ISG20 clones and the HeLa cells transfected with the empty pCiNeo vector (HeLa/pCiNeo) were infected with VSV at 0.1 MOI. 24 h later, the productions of infectious viral particles were determined as described under "Experimental Procedures." The histograms represent the log of virus yield produced by each cell population. The standard deviations were determined for three independent experiments. *C*, HeLa/pCiNeo cells and the wt-ISG20 clones were infected with 0.1 and 1 MOI of VSV, and viral productions were determined as described in *B*.

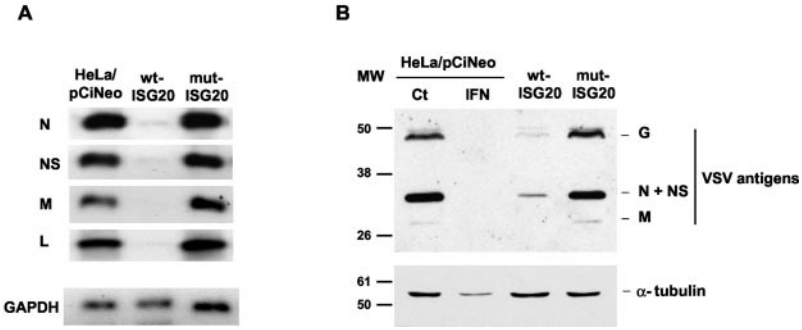

FIG. 3. **Overexpression of ISG20 inhibits viral mRNA and protein accumulation.** *A*, HeLa/pCiNeo cells and wt-ISG20- and mut-ISG20-expressing cells were infected with VSV at 1 MOI. 6 h after infection, the cells were collected, and their total RNA was extracted. RNAs (20 μg/lane) were separated on 1.2% formaldehyde-agarose gel, transferred onto a nylon membrane, and hybridized to ^{32}P-labeled cDNA probes corresponding to the four major VSV mRNAs encoding for the viral proteins N, NS, M, and G. The same blot was reprobed with a glyceraldehyde-3-phoshate dehydrogenase (*GAPDH*) probe to ensure that equal amounts of RNA were loaded onto each lane. The cDNA probes used are indicated to the *left* of the blot. *B*, HeLa/pCiNeo cells and wt-ISG20- and mut-ISG20-expressing cells were infected with VSV at 1 MOI. 16 h after infection, the cells were collected, and total protein extracts were analyzed by immunoblotting with rabbit polyclonal antibodies directed against the four major structural proteins of VSV, the nucleoproteins N and NS, the matrix protein M, and the glycoprotein G. For comparison, the level of viral protein expression in HeLa cells treated with 500 units/ml HuIFN-α2a is shown. Expression of α-tubulin was used as an invariant control.

sufficient to promote efficient protection against VSV. To determine whether the viral resistance observed was dependent on the amount of viral particles used for infections, an additional experiment was performed with 1 MOI of VSV. As expected, the amplitude of ISG20 viral protection was decreased when the cells were infected at high MOI (Fig. 2*C*). Clearly, these data strengthen our findings in transient transfection experiments and demonstrate the role of ISG20 in the antiviral actions of IFNs against VSV. Because VSV particle production was significantly higher in mut-ISG20 HeLa cells, these data confirmed the dominant-negative activity of the ExoII inactive mutant of ISG20 protein.

ISG20 Inhibits VSV RNA and Protein Accumulation—To determine whether ISG20 overexpression interfered with viral RNA expression, empty pCiNeo, wt-ISG20, and mut-ISG20 HeLa cells were infected with VSV at 1 MOI. 6 h after infection, the cells were collected, and total RNA was extracted and analyzed by Northern blot for the presence of the four major VSV mRNAs encoding the viral proteins N, NS, M, and G (25, 27). As shown in Fig. 3*A*, the expression of all the viral mRNAs tested was strongly reduced in the wt-ISG20 clone, compared with HeLa/pCiNeo cells or with the mut-ISG20-expressing cells. Hybridization with a glyceraldehyde-3-phosphate dehydrogenase probe (30) was used as an invariant control to normalize the Northern blot (Fig. 3*A*). The effect of wt-ISG20 was also confirmed by Western blot analysis using a rabbit polyclonal antibody able to detect the major structural proteins of VSV, the nucleoproteins N and NS (M_r 40,000), the matrix protein M (M_r 25,000), and the glycoprotein G (M_r 69,000) (27). In accordance with the inhibition of VSV mRNA expression, a strong reduction in VSV antigen expression was observed in the wt-ISG20 clone as compared with mut-ISG20 and HeLa/pCiNeo cells (Fig. 3*B*). The VSV antigens remained undetectable in HeLa-pCiNeo cells treated with 500 units/ml HuIFN-α2a used as positive control.

ISG20 Partly Mediates IFN Antiviral Activity against VSV—We have previously shown that ≥90% of ISG20 RNase activity was inhibited when RNase assays were performed, *in vitro*, in the presence of the inactive ExoII-mutated ISG20 protein (24). The fact that the amounts of VSV produced by

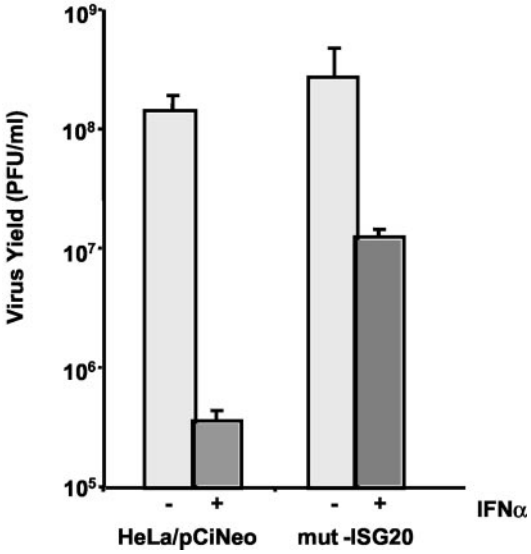

FIG. 4. **Overexpression of inactive ISG20 mutated protein acts as a dominant negative *in vivo*.** Mut-ISG20 and HeLa/pCiNeo cells were treated with 500 units/ml HuIFN-α2a for 24 h and then infected with VSV at 0.1 MOI. 24 h later, the productions of infectious viral particles were determined as described under "Experimental Procedures." The histograms represent the log of virus yield produced by each cell line. The standard deviations were determined for three independent experiments.

transiently or stably transfected HeLa cells with the mut-ISG20 construct were significantly higher than that produced by cells transfected with the empty pCiNeo vector strongly suggests that mut-ISG20 could exhibit dominant-negative effects *in vivo* (24). We took advantage of this inhibitory effect to test the contribution of ISG20 to the IFN-mediated antiviral activity. To this end, the antiviral action of IFN on mut-ISG20 HeLa cells was compared with that observed on HeLa/pCiNeo cells. The cells were treated for 16 h with 500 units/ml HuIFN-α2a and then infected with 0.1 MOI of VSV. 24 h later, the virus titers were determined as described under "Experimental Procedures." The results presented in Fig. 4 show that overexpression of mut-ISG20 protein reduced the ability of IFN to interfere with VSV infection. These data confirm that the antiviral activity of IFN against VSV infection is partly mediated by ISG20.

Overexpression of ISG20 Confers Resistance to Influenza Virus and EMCV Infections but Not to Adenovirus Infection—Because the different IFN-induced antiviral pathways described thus far present some virus specificities, we analyzed the potential ISG20 antiviral activity against two other RNA genomic viruses, the influenza virus (an orthomyxovirus) and EMCV (a picornavirus). The wt-ISG20, mut-ISG20, and HeLa/pCiNeo cells were infected with these viruses at 0.1 MOI. The viral productions were determined as described for VSV. As shown in Fig. 5*A*, a strong reduction in influenza virus production was observed with the wt-ISG20 clone but not with mut-ISG20. However, this decrease in viral multiplication was lower than the one observed with VSV. On the other hand, only a weak protective effect was observed for EMCV (Fig. 5*B*). These data show that ISG20 presents antiviral specificities among the viruses tested and suggest that this protein acts preferentially against VSV in the IFN-mediated antiviral barrier. Interestingly, the expression of mut-ISG20 did not affect the action of IFN against influenza virus and EMCV (data not shown), suggesting that the contribution of ISG20 against these viruses is probably minor in comparison with the other IFN-induced pathways.

We also addressed the question of the ability of ISG20 to

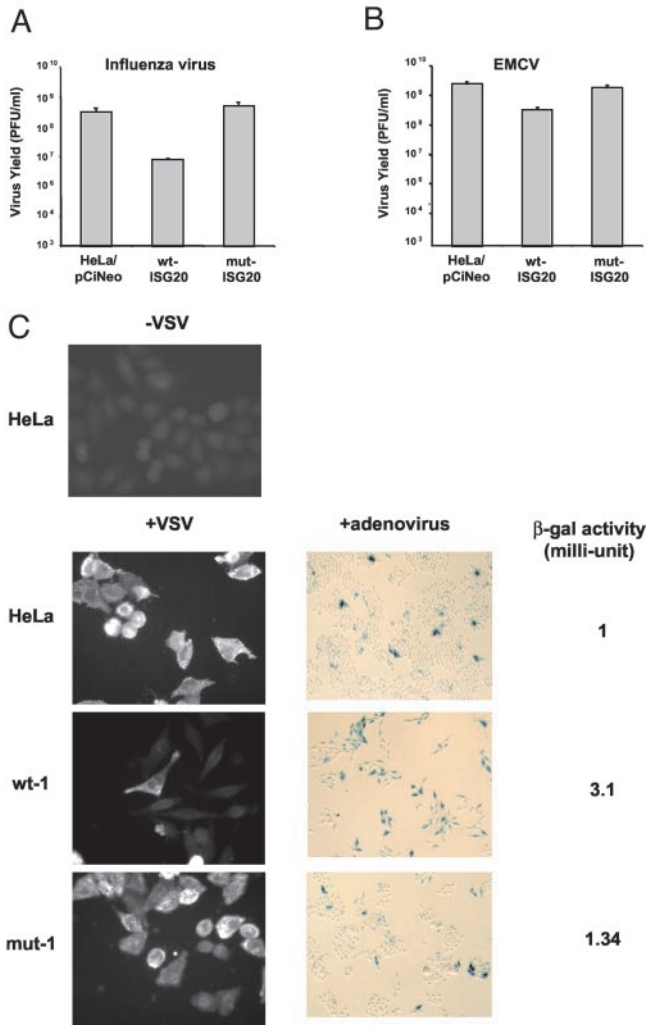

FIG. 5. **Stable constitutive expression of ISG20 protein confers resistance to influenza virus and EMCV infections but not to adenovirus infection.** HeLa/pCiNeo cells and the wt-ISG20- and mut-ISG20-expressing cells were infected with influenza virus (*A*) or EMCV (*B*) at 0.1 MOI. 24 h later, the productions of infectious viral particles were determined as described under "Experimental Procedures." The histograms represent the log of virus yield produced by each cell population. The standard deviations were determined for three independent experiments. *C*, HeLa cells and wt-ISG20 and mut-ISG20 clones were infected for 12 h with 1 MOI of VSV (+*VSV*), and VSV antigen expression was monitored by immunofluorescence with rabbit anti-VSV polyclonal antibodies. The same cells were infected by a derivative adenovirus strain bearing a β-galactosidase encoding reporter gene (+*adenovirus*) at 10 MOI. 24 h after infection, β-galactosidase-expressing cells were detected by histochemical staining and light microscopy analysis. The relative β-galactosidase activities are indicated in milliunits.

interfere with the replication of a DNA genomic virus. To this end, the HeLa/pCiNeo cells and the wt-ISG20 and mut-ISG20 clones were infected with a derivative adenovirus strain bearing a β-galactosidase reporter gene at an MOI of 10. 24 h after infection, β-galactosidase-expressing cells were detected by histochemical staining and light microscopy analysis. In addition, cellular extracts were prepared, and the β-galactosidase activity was determined as described under "Experimental Procedures." As a control, the cells were infected for 12 h with 1 MOI of VSV, and VSV antigen expression was monitored by immunofluorescence. According to our previous data, a strong inhibition of VSV antigen expression was observed in the wt-ISG20 clone (Fig. 5*C*). On the contrary, no inhibition of adenovirus protein expression was observed in wt-ISG20 HeLa cells, strengthening the idea of virus specificity for ISG20 activity.

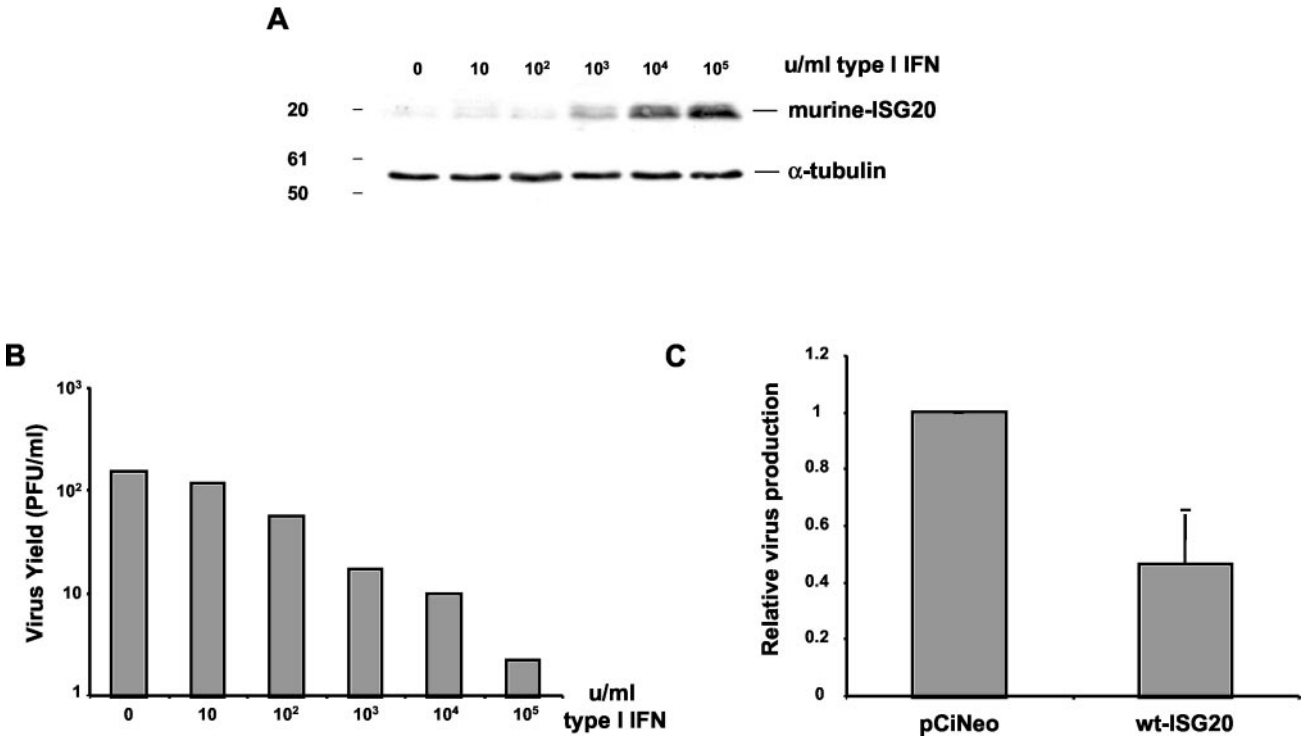

FIG. 6. **Expression of ISG20 in cells triply deficient in PKR, RNase L, and Mx proteins confers resistance to VSV infection.** *A*, triply deficient MEFs were cultured in the presence of increasing concentrations of type I IFN. Total protein extracts were analyzed by immunoblotting with a specific mouse polyclonal antibody directed against ISG20 recombinant protein. The size of the molecular weight marker (*MW*) is indicated. Expression of α-tubulin was used as an invariant control. *B*, triply deficient MEFs were cultured in the presence of increasing concentrations of type I IFN and then infected for 24 h with 0.1 MOI of VSV. Viral productions were determined as described previously. *C*, triply deficient MEFs were transiently transfected, by the LipofectAMINE Plus Reagent procedure, with 0.5 μg of wt-ISG20 or the empty pCiNeo vector. 24 h after transfections, the cells were infected with 0.1 MOI of VSV. Viral productions were determined 24 h later as described under "Experimental Procedures." The histogram represents the relative values of viral production calculated by dividing the number of viral particles obtained after transfections with the indicated constructions by the number of viral particles obtained after transfection with the empty pCiNeo vector. The standard deviations were determined for three independent experiments.

Expression of ISG20 in Cells Triply Deficient in PKR, RNase L, and Mx Proteins Confers Resistance to VSV Infection—It has been showed that MEFs triply deficient in PKR, RNase L, and Mx gene expression retain a substantial residual IFN-mediated antiviral activity against VSV, suggesting the existence of other alternative antiviral pathways (20). The ability of ISG20 to interfere with VSV multiplication prompted us to analyze the regulation of ISG20 expression by IFN and its potential antiviral activity in this cellular context. To this end, triply deficient MEFs were cultured in the presence of increasing concentrations of type I IFN and then analyzed for ISG20 protein expression. Concurrently, the cells were infected for 24 h with 0.1 MOI of VSV, and viral productions were determined as described previously. As shown in Fig. 6*A*, mouse ISG20 protein was detected with the antibody directed against its human homologue (the mouse protein shares 81% homology with the human protein) and was inducible by IFN in a dose-dependent manner. The level of the IFN-mediated antiviral activity (Fig. 6*B*) correlated with the induction of ISG20 expression (Fig. 6*A*). In addition, MEFs transiently transfected with the wt-ISG20-expressing vector exhibited a significant reduction in virus production as compared with cells transfected with the empty pCiNeo vector (Fig. 6*C*). Taken together, these data demonstrate that the induction of ISG20 expression by type I IFN does not require functional PKR, RNase L, or MX gene expressions and suggest that ISG20 might represent an alternative antiviral pathway against VSV infection.

DISCUSSION

The diverse biological activities of IFNs are thought to be mediated by the induction and activation of specific but usually overlapping sets of proteins (31). In particular, the 2-5A/RNase L system (reviewed in Refs. 2 and 13), the double-stranded RNA-dependent protein kinase (PKR) (9), and the Mx proteins (14, 15) are the three major described IFN-regulated pathways that are involved in the cellular protection against viral infections. Attempts to further resolve the contribution of each of these pathways in the antiviral activities mediated by IFN have included the establishment of genetically deficient mice (14, 20, 32–34). Interestingly, fibroblasts derived from mice triply deficient in PKR, RNase L, and Mx gene expressions were still protected by IFN against viral infections, suggesting the existence of additional IFN-induced antiviral pathways (20). The huge diversity of virus families and the fact that viruses have developed strategies to circumvent the antiviral activities of IFN implicate that mammalian cells use various alternative strategies to interfere with viral multiplication (35). We have recently isolated a human cDNA encoding a new IFN-induced gene that we have termed *ISG20* (21, 22, 29). We provided biochemical evidence that ISG20 is a processive 3′ to 5′ exonuclease specific for single-stranded RNA (24). In the present report, we analyzed the potential antiviral activity of ISG20.

We demonstrated that stable and constitutive expression of ISG20 confers resistance to VSV, influenza virus, and EMCV infection in HeLa cells, providing an alternative antiviral pathway against RNA genomic viruses. The same experiments were performed in transiently transfected cells, demonstrating that the protective effect was due to ISG20 expression and was not a characteristic of the selected clones. However, the protective efficiency of ISG20 seems to be variable even among RNA

viruses because protection against influenza virus and EMCV was less efficient than that against VSV infection. In accordance with that, an inactive mutant ISG20 protein able to inhibit ISG20 exonuclease activity *in vitro* significantly reduced the ability of IFN to block VSV but not EMCV or influenza virus developments. These data strongly suggest that ISG20 partly mediates the IFN antiviral effect against VSV, with a minor contribution against influenza virus and EMCV infections. ISG20 did not seem to interfere with the replication of a derivative adenovirus strain bearing a β-galactosidase-encoding reporter gene. This suggests that single-stranded RNA genomic viruses might be preferential targets for ISG20. These data are not surprising because the effectiveness with which the host's antiviral response can clear a virus infection indicates that cooperation between several antiviral pathways is required. Each of these pathways affects different stages of the viral life cycle (2, 9, 13, 19, 36). However, the virus specificity of each of these pathways is not clearly established and seems dependent on the cell type or the animal model used (32, 37–39). Thus, additional studies with several other RNA and DNA viruses are needed to precisely clarify the viral specificities of ISG20.

Our dominant-negative experiments suggest that the antiviral action of ISG20 is mediated by its exonuclease activity. Throughout its 3′ to 5′ exonuclease, it is conceivable that ISG20 might affect viral development by degrading viral RNA. However, we cannot exclude that ISG20 acts indirectly on cellular factors required for viral replication or transcription. Like ISG20 and RNase L, some cellular and extracellular ribonucleases appear to be major contributors to the protection against various pathogens including viruses and bacteria. Because dsRNAs are formed in almost all viral infections, they represent preferential targets for the ribonuclease-mediated antiviral effect. Indeed, it has been shown that dsRNA duplexes can be hyper-edited by members of the adenosine deaminase enzyme family, resulting in up to 50% adenosine to inosine conversion (40). Hyper-edited dsRNA are specifically cleaved by a cytoplasmic endoribonuclease that requires an RNA structure fitted to hyper-edited RNA (41). The fact that the cytoplasmic isoform of adenosine deaminase (ADAR1) is inducible by IFN (42) lent weight to the idea that this process provides an efficient mechanism to remove long, uninterrupted dsRNAs frequently associated with infection by viruses. In addition, adenosine to inosine editing dramatically changes the stability of dsRNA structures, resulting in a stronger vulnerability to attacks by single-stranded RNAses (43). In particular, inosine-containing single-stranded RNA and unwinding dsRNA edited by ADAR1 have been reported to be degraded at a highly accelerated rate by a specific 3′ to 5′ exonuclease termed I-RNase (44). The authors speculated that I-RNase, in concert with ADAR1, might form part of a novel antiviral defense mechanism that acts to degrade dsRNA. Some extracellular ribonucleases also display antiviral properties. The eosinophil-derived neurotoxin protein (EDN or RNase 2) and the eosinophil cationic protein (ECP or RNase 3), members of the RNase A family found in secretory granules of human eosinophilic leukocytes, reduce the infectivity of certain RNA viruses including respiratory syncytial virus (45) and human immunodeficiency virus (46). These activities are mediated through an RNase-dependent process. In the same way, human onconase has been shown to act as a ribonuclease-dependent antiviral agent (47). Surprisingly, nothing is known about the contribution of deoxyribonucleases in the control of viral development in particular against DNA viruses.

More generally, the control of RNA turnover is involved in the regulation of critical functions such as cell cycling, apoptosis, and stress response. The fact that all these functions appear to be modulated by IFN and the fact that IFN can regulate the expression of cellular genes at the RNA level (48, 49) suggest that control of RNA stability may play a major role in the mechanism of IFN action. Thus, the identification of cellular targets of ISG20 remains a main challenge for comprehension of the molecular mechanism of IFN action. However, how ISG20 can specifically degrade particular viral or cellular RNAs remains unclear. Indeed, RNases are typically present in very low amount in cells associated with a specific inhibitor or are present in an inactive latent form requiring the presence of a specific activator. This is the case for RNase L, whose activation requires the presence of oligoadenylates synthesized by 2′-5′ oligoadenylate synthetase in response to replicating dsRNA forms of viruses such as EMCV (12, 50, 51). It is conceivable that such a mechanism could be involved in a local activation of ISG20 preventing cell toxicity.

Recently, monitoring of global gene expression of immune cells using DNA microarrays revealed two clusters of IFN-induced genes that are coordinately expressed (52). One of them, termed IFN-2, contains genes that promote resistance to viral infection and reflects a coordinated effort by cells to escape viral control, including RNase L, PKR, Mx, and ISG20 genes. Interestingly, we showed that mouse ISG20 protein was inducible by type I IFN in MEFs triply deficient in RNase L, PKR, and Mx gene expressions. In addition, these cells transiently transfected with the wt-ISG20-expressing vector exhibited a protection against VSV infection. These data greatly strengthened the role of ISG20 as an alternative antiviral pathway. More recently, ISG20 was shown to be up-regulated during activation programs induced in human macrophages by some bacterial strains, providing a more general role of ISG20 against different kinds of pathogens (53, 54). Additional studies are needed to precisely determine the biological contribution of ISG20 in these processes.

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ISG20, a New Interferon-induced RNase Specific for Single-stranded RNA, Defines an Alternative Antiviral Pathway against RNA Genomic Viruses

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