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18. Amino acid residue numbers refer to the rat brain type IIA sequence reported in (*8*). Mutant Na^+ channel constructs are referred to by the natural amino acid followed by the residue number (or numbers) and the mutant amino acid. For example, S1506A refers to mutation of the wild-type serine at position 1506 to an alanine. Abbreviations for the amino acid residues are: A, Ala; D, Asp; E, Glu; G, Gly; K, Lys; L, Leu; Q, Gln; and S, Ser.
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20. In whole cell recordings from cells that expressed mutant Na^+ channel subunits S1506E and S1506D, the voltage dependence of Na^+ channel activation and steady-state inactivation were both shifted to values 20 mV more positive than the values obtained for wild-type channels. Therefore, we measured peak Na^+ currents at 0 mV rather than −20 mV.
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NF-κB Activation by Ultraviolet Light Not Dependent on a Nuclear Signal

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Exposure of mammalian cells to radiation triggers the ultraviolet (UV) response, which includes activation of activator protein–1 (AP-1) and nuclear factor kappa B (NF-κB). This was postulated to occur by induction of a nuclear signaling cascade by damaged DNA. Recently, induction of AP-1 by UV was shown to be mediated by a pathway involving Src tyrosine kinases and the Ha-Ras small guanosine triphosphate–binding protein, proteins located at the plasma membrane. It is demonstrated here that the same pathway mediates induction of NF-κB by UV. Because inactive NF-κB is stored in the cytosol, analysis of its activation directly tests the involvement of a nuclear-initiated signaling cascade. Enucleated cells are fully responsive to UV both in NF-κB induction and in activation of another key signaling event. Therefore, the UV response does not require a signal generated in the nucleus and is likely to be initiated at or near the plasma membrane.

Exposure of mammalian cells to UV and other DNA-damaging agents activates the UV response (*1*). Two transcription factors that execute part of this induction response are AP-1 (*2*, *3*) and NF-κB (*2*). The latter may be responsible for stimulation of human immunodeficiency virus–type 1 (HIV-1) transcription (*2*, *4*) and thereby cause its activation in skin cells (*5*). However, it was also proposed that UV-mediated DNA damage activates HIV-1 by nonspecific decondensation of chromatin (*6*), an unlikely mechanism given the established role of NF-κB in HIV-1 induction (*7*). An unanswered problem is the mechanism by which UV activates NF-κB. Because NF-κB is stored as an inactive cytoplasmic complex by way of interaction with IκB (*8*), its activation requires the transfer of a UV-generated signal to that subcellular compartment. The UV response was proposed to be mediated by an intranuclear signaling cascade, elicited by damage to DNA (*1*). If this mechanism is involved in the activation of NF-κB by UV, then a nuclear signal should be transferred to the cytoplasm to induce dissociation of the NF-κB:IκB complex.

We demonstrated that exposure of HeLa cells to UV rapidly activates Src-family tyrosine kinases, followed by activation of the small guanosine triphosphate (GTP)–binding protein (G protein) Ha-Ras and the cytoplasmic serine-threonine kinase Raf-1 (*9*). This cascade increases the phosphorylation of c-Jun, an important component of AP-1 (*10*), on sites that stimulate its transcriptional activity (*9*). Because c-Src and Ha-Ras are primarily associated with the plasma membrane (*11*), we proposed that the signaling cascade leading to activation of AP-1 by UV is initiated at the plasma membrane (*9*). However, a very rapid transfer of a nuclear-generated signal to the plasma membrane could not be excluded. We now demonstrate that tyrosine kinases and Ha-Ras are also involved in activation of NF-κB by UV. With the use of enucleated cells, we show that activation of NF-κB and an early signaling event caused by UV do not require a nuclear signal. Therefore, the UV signaling cascade activating both NF-κB and AP-1 is initiated at or near the plasma membrane and is not elicited by DNA damage in the nucleus.

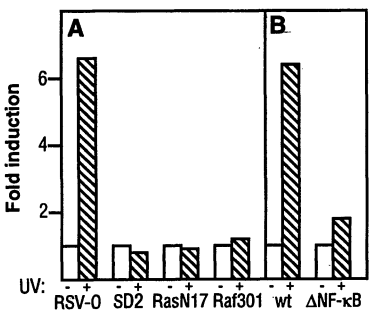

Fig. 1. Inhibition of HIV-1 promoter activation by dominant negative alleles of v-*src*, Ha-*ras*, and *raf-1*. (**A**) HeLa S3 cells were cotransfected with 3 μg of (−453/+80)HIV-LUC per plate, together with either the expression vector alone (RSV-O; 10 μg) or expression vectors encoding dominant interfering v-*srcSD2* (7 μg), Ha-*rasN17* (0.34 μg), and *raf301* (10 μg) alleles. The total amount of expression vector was kept constant at 10 μg with RSV-O. One-half of the cultures were exposed to UVC (40 J/m²) 2 hours after transfection. Luciferase activity was determined (*9*) 22 hours later. The results shown are averages of two separate experiments and are presented as fold induction above the basal (−453/+80)HIV-LUC expression, which averaged 70 U per microgram of cell protein. (**B**) HeLa S3 cells were cotransfected with 3 μg of (−453/+80)HIV-LUC or ΔNF-κB(−453/+80)HIV-LUC (*7*) per plate. One-half of the cultures were exposed to UVC (40 J/m²) 2 hours after the transfection, and LUC activity was determined 22 hours later. Shown are the averages of two separate experiments. Basal expression in these experiments averaged 21 U per microgram of protein.

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The NF-κB–dependent (−453/+80) HIV-luciferase (LUC) reporter (*2, 7*) was cotransfected with vectors encoding dominant negative alleles of v-*src*, Ha-*ras*, and *raf-1* (*12*) into HeLa cells. Whereas an empty expression vector (RSV-O) had no effect on HIV-LUC expression, each of the three dominant negative alleles prevented induction by UV (Fig. 1A). This inhibition is specific because none of the dominant negative alleles inhibited constitutive promoters (*9*) or basal HIV-LUC expression. Induction of HIV-LUC by UV requires intact NF-κB binding sites (*2*) (Fig. 1B).

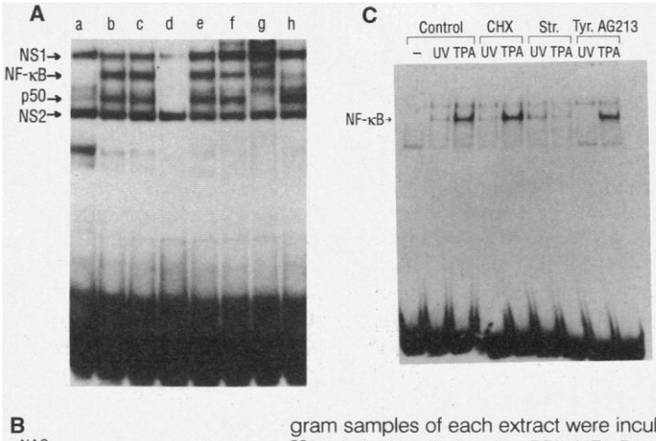

Fig. 2. Characterization of NF-κB induction by UV. (**A**) HeLa S3 cells were serum starved in 0.1% fetal calf serum (FCS) for 16 hours and then either kept in Dulbecco's minimum essential medium (DMEM) plus 0.1% FCS with no further treatment (lane a) or exposed to UVC (40 J/m^2) (lanes b through h). After 5 hours the cells were harvested and nuclear extracts were prepared (*15*). Five-microgram samples of each extract were incubated with 10,000 cpm of a ^{32}P-labeled palindromic (PD) NF-κB probe (*29*). Electrophoretic mobility shift assays were done as described (*15*). The specificity of binding was determined by competition experiments using either 2 ng of an AP-1 binding site oligonucleotide (lane c) or 2 ng of unlabeled PD oligonucleotide (lane d). The composition of the NF-κB complexes was determined by incubation of the extracts with 1 μl of either normal rabbit serum (lane e), polyclonal antibody to p98 and p55 (lane f), polyclonal antibody to p105 and p50 (lane g), or polyclonal antibody to p65 (lane h). The various antibodies and their specificities were described (*15*). The migration positions of the p50:p65 (NF-κB) and p50:p50 complexes and two nonspecific (NS) protein-DNA complexes are shown. (**B**) HeLa S3 cells were serum starved for 16 hours and incubated in the absence or presence of 40 mM NAC for 1 hour followed by no further treatment (−) or exposure to either UVC (40 J/m^2) or TPA (100 ng/ml). Cell extracts were prepared after 2 hours (UV) or 1 hour (TPA or control), separated by SDS-PAGE (30 μg of protein per lane), transferred to PVDF membrane, and immunoblotted with polyclonal antibody to IκB-α. The migration position of IκB-α is indicated. NS, a nonspecific cross-reacting protein. (**C**) HeLa cells were grown to confluency, serum starved for 16 hours, and treated with either 20 μM tyrphostin (Tyr.) AG213, 100 nM staurosporine (Str.), or cycloheximide (CHX; 10 μg/ml) for 1 hour before exposure to TPA (100 ng/ml) or UV (40 J/m^2) as indicated. Five hours later the cells were harvested and nuclear extracts were prepared. Five micrograms of each extract were incubated with 10,000 cpm of ^{32}P-labeled κB oligonucleotide (*8*) and resolved by electrophoresis on a native gel.

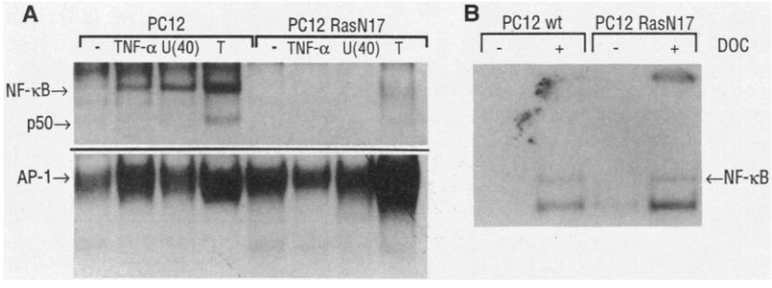

Fig. 3. Induction of NF-κB binding activity by UV and TNF-α requires Ha-Ras activity. (**A**) Wild-type PC12 cells and PC12 cells stably transfected with Ha-*rasN17* (*20*) were serum starved for 16 hours and then exposed to either TPA (100 ng/ml; T), UV (40 J/m^2) [U(40)], TNF-α (10 ng/ml), or kept in DMEM with 0.1% FCS and 0.1% donor horse serum(−). Five hours later cells were harvested and nuclear extracts were prepared. Ten micrograms of each extract were incubated with 10,000 cpm of ^{32}P-labeled κB or AP-1 (*3*) oligonucleotides and resolved by electrophoresis on native gels. Both the top and the bottom panels show only the most relevant segment of each autoradiogram corresponding to the NF-κB and AP-1 complexes, respectively. (**B**) Ten-microgram samples of cytosolic extract from uninduced wild-type PC12 or Ha-*rasN17*–transfected PC12 cells were treated with 0.4% sodium deoxycholate and 1% NP-40 and analyzed for NF-κB binding activity by a mobility-shift assay.

Ultraviolet irradiation of HeLa cells also induced NF-κB binding activity (Fig. 2A) whose major species contained the p65 subunit (*13*), because preincubation with antiserum to p65 inhibited DNA binding (Fig. 2A, lane h). The second, faster migrating NF-κB species is likely to be a homodimer of the p50 subunit (*14*) because its mobility was retarded by antiserum to p50 (Fig. 2A, lane g). The same pattern of immunochemical reactivity was found for NF-κB complexes induced by the phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) (*15, 16*). In agreement with previous findings with TPA as an inducer (*17*), UV irradiation caused degradation of IκB-α (Fig. 2B). This degradation was inhibited by preincubation with *N*-acetylcysteine (NAC), which inhibited induction of NF-κB binding activity by UV or TPA (*18*).

Inhibition of HIV-LUC induction by dominant negative v-*src* suggested that NF-κB activation by UV involves tyrosine kinases. The specific tyrosine kinase inhibitor tyrphostin AG213 (*19*) blocked induction of NF-κB binding activity by UV but not by TPA (Fig. 2C). In contrast, another inhibitor, staurosporine, inhibited induction of NF-κB by TPA but not by UV. This suggests that either two different pathways lead to induction of NF-κB or that UV and TPA affect different steps of the same signaling cascade. Cycloheximide did not block induction of NF-κB by either agent, suggesting that both responses involve activation of preexisting NF-κB complexes. NF-κB activity was induced within 30 min of UV exposure; maximal induction occurred at 2 hours after exposure (*18*).

To further examine the role of Ha-Ras, we used a PC12 cell line stably transfected with Ha-*rasN17*, a dominant negative allele of c-Ha-*ras* (*20*). Exposure of these cells to UV did not result in induction of NF-κB activity, whereas wild-type PC12 cells were responsive (Fig. 3A). The mutant cell line was also refractory to the cytokine tumor necrosis factor–α (TNF-α), whereas its response to TPA was only partially attenuated. In both cell lines equal amounts of NF-κB binding activity were revealed by treatment of cytosols with deoxycholate and NP-40 (Fig. 3B), which dissociate the NF-κB:IκB complex (*8*). The quality of the extracts was controlled by measurement of AP-1 binding activity, which was only slightly elevated after 5 hours of exposure to either UV or TNF-α (Fig. 3A). TPA increased AP-1 binding activity in both cell lines, indicating that, consistent with previous findings in other cell types (*21*), Ha-Ras may not be involved in induction of AP-1 by TPA.

Exposure to UV activates Ha-Ras, as measured by GTP binding (*9*). One conse-

quence of Ha-Ras activation is increased phosphatidylcholine (PC) turnover (*22*). Exposure of wild-type PC12 cells to UV resulted in a significant increase in PC turnover as measured by the release of phosphocholine, whereas no significant response could be detected in Ha-*rasN17*–transfected PC12 cells (*23*).

To rigorously establish the involvement of Ha-Ras in UV induction of HIV-LUC, we cotransfected this reporter with expression vectors encoding normal and oncogenically activated Ha-Ras and activated Raf-1 into Ha-*rasN17*–transfected PC12 cells. Whereas expression of normal c-Ha-Ras restored UV inducibility of HIV-LUC (Fig. 4A), expression of oncogenically activated Ha-Ras and Raf-1 led to its constitutive activation (Fig. 4B).

Thus, similar to AP-1, UV induces NF-κB by activating a signaling cascade involving Src tyrosine kinases and the Ha-Ras small G protein. Because both c-Src and Ha-Ras are associated with the inner face of the plasma membrane (*24*) and are rapidly activated by UV (9), it is likely that the UV response is initiated at this compartment. However, other investigators suggested that a signal generated by UV-damaged DNA in the nucleus is rapidly transferred to the cytosol, leading to downstream events (*25*). To directly test the involvement of the nucleus in the UV response, we made use of HeLa cells enucleated by cytochalasin B treatment (*26*). Exposure to UV induced amounts of NF-κB binding activity in cytoplasts similar to that in intact cells (Fig. 5A). The efficiency of enucleation is demonstrated by the loss of AP-1 binding activity. Staining with a karyophilic dye revealed that enucleation efficiency was at least 95% (*26*). Recently, we identified a protein kinase, JNK, which produces phosphorylation of c-Jun in response to UV irradiation, whose activity is greatly induced in response to UV exposure (*27*). The JNK activity is induced by UV exposure of cytoplasts as well as in intact cells (Fig. 5B).

These experiments conclusively rule out the involvement of a nuclear signal generated by DNA damage in induction of either NF-κB or AP-1 by UV. Whereas we have not eliminated the possibility that damaged mitochondrial DNA is involved in triggering the UV response, we consider this unlikely. If the system that leads to the induction of either JNK or NF-κB monitors the total amount of DNA damage, a considerable reduction in the extent of induction of either activity should have occurred after enucleation, because most of the cellular DNA is lost. Because NF-κB is the major transcription factor responsible for induction of HIV-1 promoter activity by UV (*2*) and by other inductive signals (*7*), it is unlikely that UV activates HIV-1 by means of chromatin decondensation as proposed (6). If UV induction is an important contributor to HIV-1 activation in dendritic cells in the skin (*5*), it may be possible to attenuate this response by inhibitors of either Src or Ha-Ras activation.

We demonstrate that activation of Ha-Ras is also required for the induction of NF-κB by TNF-α. Based on the inhibition of NF-κB by NAC and other antioxidants, it was previously suggested that TNF-α induces NF-κB activity through production of reactive oxygen species (*28*). Our results indicate that if reactive oxygen species mediate NF-κB activation by either UV or TNF-α, they must act upstream of Ha-Ras rather than on the NF-κB:IκB complex itself.

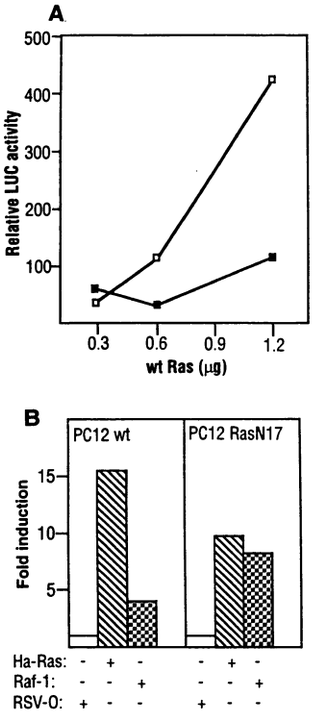

Fig. 4. Reconstitution of UV responsiveness in Ha-*rasN17*–transfected PC12 cells. (**A**) Ha-*rasN17*–transfected PC12 cells were cotransfected with 3 μg of (−453/+80)HIV-LUC per plate together with the indicated amounts of an expression vector encoding nonactivated Ha-Ras (pZIPNeo-Ras) (*30*). The cells were either not exposed (■) or exposed (□) to UVC (40 J/m^2) 2 hours after the transfection and 21 hours later were harvested for determination of LUC activity. The results shown are averages of two experiments and are presented as LUC activity per microgram of cell protein. (**B**) Wild-type PC12 cells and Ha-*rasN17*–transfected PC12 cells were cotransfected with 3 μg of (−453/+80)HIV-LUC per plate, together with expression vectors encoding oncogenically activated Ha-Ras (0.5 μg) or Raf-1 (10 μg) (*12*). The total amount of expression vector was kept at 10 μg with RSV-O. The cells were harvested 20 hours later for determination of LUC activity. The results shown are averages of two experiments and are presented as fold induction above the basal HIV-LUC expression, which averaged 62 U per microgram of protein for Ha-*rasN17*–transfected PC12 cells and 8.4 U per microgram of protein for wt PC12 cells.

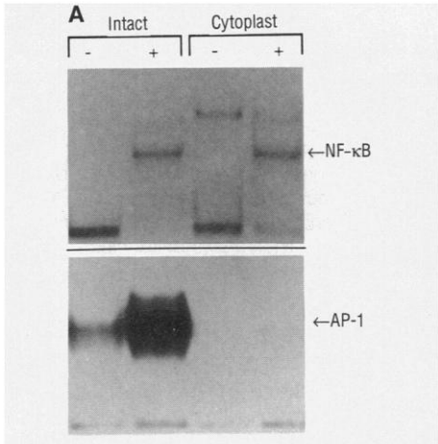

Fig. 5. Induction of NF-κB binding activity and JNK activity in enucleated cells. (**A**) NF-κB. Cytoplasts were separated from intact cells after cytochalasin B treatment and ultracentrifugation as described (*26*). Adherent cytoplasts or intact cells were either left untreated (−) or exposed to UVC (40 J/m^2) (+). After 4 hours the cells and cytoplasts were harvested, and total extracts were prepared as described (*15*). Ten micrograms of each extract were incubated with 10,000 cpm of ^{32}P-labeled κB (upper panel) or AP-1 (lower panel) and analyzed by mobility-shift assays. The migration positions of the NF-κB and AP-1 protein-DNA complexes are indicated. (**B**) Adherent cytoplasts and whole cells obtained as described above were left untreated (−) or were exposed to UVC (40 J/m^2) (+). Thirty minutes later the cells and cytoplasts were harvested and total extracts were prepared. Thirty micrograms of each extract were assayed for JNK activity with a GST–c-Jun(AA1–223) fusion protein as a substrate (*27*). The phosphorylated protein was analyzed by gel electrophoresis and autoradiography.

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27. M. Hibi, A. Lin, T. Smeal, M. Karin, *Genes Dev.*, in press; 30 μg of total cell extract were incubated for 3 hours at 4°C with a glutathione-S-transferase (GST)–c-Jun(1–223) fusion protein coupled to glutathione-agarose beads, followed by five washes with HBB [20 mM Hepes (pH 7.7), 50 mM NaCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% Triton X-100]. The resuspended beads were then used to phosphorylate recombinant c-Jun (10 ng) by incubation for 30 min at 30°C in 20 mM Hepes (pH 7.5), 20 mM β-glycerophosphate, 20 mM *p*-nitrylphenyl phosphate, 10 mM $MgCl_2$, 1 mM dithiothreitol, 50 μM Na_3VO_4, 2 μCi of γ-^{32}P–labeled adenosine triphosphate (ATP), and 20 μM unlabeled ATP. The reaction was stopped by the addition of SDS gel loading buffer, boiled for 5 min, and was run on a 10% SDS polyacrylamide gel.
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Inhibition of Viral Replication by Interferon-γ–Induced Nitric Oxide Synthase

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Interferons (IFNs) induce antiviral activity in many cell types. The ability of IFN-γ to inhibit replication of ectromelia, vaccinia, and herpes simplex-1 viruses in mouse macrophages correlated with the cells' production of nitric oxide (NO). Viral replication was restored in IFN-γ–treated macrophages exposed to inhibitors of NO synthase. Conversely, epithelial cells with no detectable NO synthesis restricted viral replication when transfected with a complementary DNA encoding inducible NO synthase or treated with organic compounds that generate NO. In mice, an inhibitor of NO synthase converted resolving ectromelia virus infection into fulminant mousepox. Thus, induction of NO synthase can be necessary and sufficient for a substantial antiviral effect of IFN-γ.

Several cytokines, including interferons (IFN-α, -β, -γ, and -ω) and tumor necrosis factors (TNF-α and -β) display antiviral activity (*1*). The antiviral effects of the IFNs have been attributed in part to their induction of (i) P1 kinase, which phosphorylates and inactivates eukaryotic protein synthesis initiation factor eIF-2α; (ii) 2′,5′-oligoadenylate synthetase, whose products activate a latent endoribonuclease; (iii) indoleamine 2,3-dioxygenase, which depletes cell cultures of tryptophan; (iv) Mx proteins, which inhibit influenza and vesicular stomatitis viruses; and (v) the 9-27 protein, which binds to the Rev-responsive element of human immunodeficiency virus (HIV) (*1*). Nevertheless, the mechanisms of action of IFNs remain incompletely understood.

The antimicrobial and antiproliferative actions of cytokines such as IFN-γ are undergoing reassessment in light of their ability to induce the expression of *iNOS*, a gene encoding an isoform of nitric oxide synthase (NOS) that produces large amounts of the radical gas, NO, from a guanidino nitrogen of L-arginine (*2*, *3*). Induction of *iNOS* contributes to IFN-γ's antiproliferative actions (*4*) and to its enhancement of macrophage cytotoxicity toward tumor cells, bacteria, fungi, protozoa, and helminths (*2*, *3*). The inducibility of high-output NO production among many other cell types, such as hepatocytes, smooth and cardiac myocytes, keratinocytes, endothelium, mesangial cells, tumor cells, and some fibroblasts (*2*, *5*), prompted the hypothesis that the induction of *iNOS* might also defend the host against the one class of pathogens that can infect all nucleated cells—viruses (*2*).

To test this hypothesis, we first focused on ectromelia virus (EV). The replication of this orthopoxvirus in C57BL/6 mice is restricted by IFN-γ (*6*), yet EV may inhibit both the P1 kinase and the 2′,5′-oligoadenylate synthetase pathways (*7*). In vitro, IFN-γ inhibits EV replication in mouse RAW 264.7 macrophage-like cells and in primary mouse macrophages but not in L929 mouse fibroblasts or 293 human renal epithelial cells (Fig. 1A). Thus, an additional, IFN-γ–inducible antiviral pathway is likely to exist and be selectively ex-

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