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Vaccinia Virus Blocks Gamma Interferon Signal Transduction: Viral VH1 Phosphatase Reverses Stat1 Activation

PILAR NAJARRO,[1] PAULA TRAKTMAN,[2] AND JOHN A. LEWIS[1]*

Department of Anatomy & Cell Biology, SUNY Downstate Medical Center, Brooklyn, New York 11203,[1] and Department of Microbiology & Molecular Genetics, Medical College of Wisconsin, Milwaukee, Wisconsin 53226[2]

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We have analyzed the effects of vaccinia virus (VV) on gamma interferon (IFN-γ) signal transduction. Infection of cells with VV 1 to 2 h prior to treatment with IFN-γ inhibits phosphorylation and nuclear translocation of Stat1 and consequently blocks accumulation of mRNAs normally induced by IFN-γ. While phosphorylation of other proteins in the IFN-γ pathway was not affected, activation of Stat1 by other ligand-receptor systems was also blocked by VV. This block of Stat1 activation was dose dependent, and although viral protein synthesis was not required, entry and uncoating of viral cores appear to be needed to block the accumulation of phosphorylated Stat1. These results suggest that a virion component is responsible for the effect. VV virions contain a phosphatase (VH1) that is sensitive to the phosphatase inhibitor Na_3VO_4 but not to okadaic acid. Addition of Na_3VO_4 but not okadaic acid restored normal Stat1 phosphorylation levels in VV-infected cells. Moreover, virions containing reduced levels of VH1 were unable to block the IFN-γ signaling pathway. In vitro studies show that the phosphatase can bind and dephosphorylate Stat1, indicating that this transcription factor can be a substrate for VH1. Our results reveal a novel mechanism by which VV interferes with the onset of host immune responses by blocking the IFN-γ signal cascade through the dephosphorylating activity of the viral phosphatase VH1.

Gamma interferon (IFN-γ) plays a key role in host defense (67). It regulates the adaptive immune response by enhancing major histocompatibility complex (MHC) class I expression in most cells and inducing MHC class II expression in antigen-presenting cells and endothelial cells. It is also the major physiological activating factor of macrophages and is responsible for induction of nonspecific immune responses. IFN-γ acts synergistically with tumor necrosis factor (cytotoxic activity/inflammatory response) and IFNα/β (antiviral activity). IFNs are essential and functionally nonredundant in successful host responses to certain viruses (48).

IFN-γ exerts its action through its ability to bind to the IFN-γ receptor (IFN-γR) and induce dimerization of receptor α and β subunit pairs to form a heterotetramer (18, 19). The IFN-γR α and β chains associate with Janus protein kinases Jak1 and Jak2, respectively (34, 37, 47, 65). Ligand-induced association of the receptor subunits allows these kinases to phosphorylate the IFN-γRα. The phosphorylated tyrosine (P-Tyr) and adjacent residues constitute a docking site for the sh2 domain of Stat1 (p91) (16, 22), which is present in the cytoplasm as a latent transcription factor. Once bound to the receptor-Jak complex, Stat1 is phosphorylated (p91-P) (57) and subsequently dissociates from the receptor and homodimerizes, by a process that is not fully understood, to form the IFN-γ activation factor GAF. The IFN-γ activation factor is translocated to the nucleus, where it is able to bind to the IFN-γ activation sequence—GAS—in the promoters of genes whose expression is induced by IFN-γ (57), including IFN-γ itself and Stat1 (11, 24, 50). Maximal transcriptional activity of Stat1 requires both tyrosine and serine phosphorylation (66).

Termination of the signaling induced by IFN-γ has been reported to be mediated at least in part by internalization and degradation of receptor-ligand complexes (15, 17) and by ubiquitination and proteosomal degradation of phosphorylated Stat1 (36). The role of phosphatases in the downregulation of IFN-γ-stimulated gene expression has also been demonstrated by studies in which Stat1 phosphorylation was induced and/or prolonged in the presence of phosphatase inhibitors (10, 28). Thus, dephosphorylation may act by competing with the pathway that leads to Stat1 phosphorylation or by turning off an established Stat1 signal (13, 28, 38).

Vaccinia virus (VV), which belongs to the Poxviridae family, is characterized by its complexity and ability to replicate in the cytoplasm of host cells. It has a genome composed of a single 200-kbp, linear double-stranded DNA (dsDNA) molecule with hairpin termini and a complex virion that, in addition to the structural polypeptides, contains multiple virus-encoded enzymes. These include RNA polymerase and enzymes needed to produce polyadenylated, capped, and methylated mRNAs, two DNA-dependent ATPases, a DNA topoisomerase I, two protein kinases, and a phosphatase.

In the early steps of VV infection, the virus undergoes uncoating in a two-step process (31) which releases viral cores, and later viral DNA, into the cytoplasm (7, 35). Primary uncoating occurs after fusion of the virion with the plasma membrane, and synthesis of early mRNAs can be detected 20 min after initiation of synchronous infection (14). These mRNAs encode early proteins that act as growth factors, immune defense molecules, and enzymes and factors required for DNA replication and intermediate transcription. A second uncoating step results in the removal of core proteins and release of viral DNA from the virion. This step is blocked by inhibitors of

* Corresponding author. Mailing address: Dept. Anatomy & Cell Biology, Box 5, SUNY Downstate Medical Center, 450 Clarkson Ave., Brooklyn, NY 11203. Phone: (718) 270-2215. Fax: (718) 270-3732. E-mail: jlewis@netmail.hscbklyn.edu.

protein synthesis (32). Uncoating allows replication of the viral genome and transcription of the intermediate mRNAs in discrete areas of the cytoplasm known as viral factories.

In the course of evolution, poxviruses have developed different mechanisms to overcome host defense systems (61). VV has evolved mechanisms to neutralize the activity of several host cytokines, including IFN (51, 58, 59). VV open reading frames (ORFs) E3L and K3L encode intracellular proteins that block the IFN-induced inhibition of protein synthesis by inhibiting the activation and/or action of the dsRNA-activated kinase PKR (6, 8). The activity of IFNs is also blocked by release of VV-encoded, soluble receptor analogues for the IFN-α/β receptor, B18R (9, 40), and the IFN-γ receptor, B8R (1, 46). These molecules inhibit binding of the ligand to cell surface receptors and block IFN action.

The VV H1 gene product (VH1) was the first protein identified as a dual-specificity protein tyrosine phosphatase (DS-PTPase) (23). In VV the H1L gene is localized in the central region of the viral genome (ORF H1), a region that is highly conserved among poxviruses and within which many of the proteins essential for virus survival are clustered. The inability to segregate null mutants for VV H1L or myxoma virus I1L phosphatases indicates that the phosphatase is essential for virus viability in tissue culture (42, 45). VH1 is characterized by its resistance to okadaic acid (OA) and its sensitivity to sodium vanadate (23). It is expressed late in the virus life cycle and is packaged within the virion at about 200 molecules per viral particle (42). Analysis of the localization of the encapsidated VH1 showed that after permeabilization of the viral particles, half of the activity remains core associated, and the other half is released to the supernatant fluid. Virus-encoded phosphatases are likely to be involved in the life cycle of the virus by mediating processes obligatory for replication and propagation but may also be involved in nonessential events that influence host range and virulence.

The results presented here provide strong evidence that the VH1 phosphatase acts to block IFN-γ signaling, providing another mechanism by which VV can evade host defenses.

MATERIALS AND METHODS

Tissue culture, IFN and antibodies. HeLa cells were grown in minimal essential medium (MEM), 4 g of glucose per liter, supplemented with 10% neonatal calf serum (NCS). HeLa spinner cells (HeLa-S_3) were grown in suspension using modified MEM for suspension cultures (S-MEM), 1 g of glucose per liter, and 10% NCS. All culture media were supplemented with antibiotics (penicillin, 5×10^5 U/liter, and streptomycin, 100 mg/liter). The cells were replated after trypsin-EDTA treatment twice per week for a maximum of 14 passages. Human recombinant IFN-γ was purchased from Boehringer Mannheim and titrated to 100 IU/μl. Monoclonal antibodies (MAbs) anti-Stat1, anti-Jak1, anti-Jak2, and anti-P-Tyr (PY20) were from Transduction Laboratories, Lexington, Ky. Early VV protein l3 was detected using a polyclonal rabbit antiserum. Indirect immunofluorescence was detected using tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse or goat anti-rabbit immunoglobulin (IgG) from Jackson Immuno Research Lab, Inc.

Viruses. The Western Reserve (WR) strain of VV was propagated in HeLa-S_3 cells and purified according to Joklik (30). Radioactive labeled virus was prepared similarly except that [^{3}H]thymidine (40 to 60 Ci/mmol) was added after adsorption at a final concentration of 1 μCi/ml. Stocks of the VH1 inducible mutant VV strain (vindH1) were prepared as described previously (42) by growth in the presence or absence of IPTG (isopropylthiogalactopyranoside). Titration of viral stocks was performed as described previously (40).

Virus was adsorbed to cells by incubation on ice for 1 h while agitating occasionally. At time zero postinfection (p.i), unbound virus was washed away with phosphate-buffered saline (PBS), low-serum medium (MEM with 2% NCS) was added to the cells, and infection was continued at 37°C.

Purified virus was UV-irradiated using a germicidal lamp as described by Bablanian et al. (4). Briefly, virus stock was diluted in PBS, and 1-ml aliquots were placed into 35-mm petri dishes. Incident energy was monitored with a UV meter (Blak-Ray; Ultraviolet Products, San Gabriel, Calif.). During irradiation, samples were placed on ice and gently agitated on an orbital shaker.

Determination of viral uncoating. After adsorption and infection of HeLa cells with [^{3}H]thymidine-labeled virus, cells were scraped into cold hypotonic buffer (10 mM KCl, 10 mM Tris-HCl [pH 7.4], 1.5 mM $MgCl_2$). Samples were freeze-thawed once to facilitate membrane rupture and then Dounce homogenized until >90% of cells were disrupted. Samples were incubated with DNase I (EC 3.1.21.1; Sigma) at 200 μg/ml and 10 mM magnesium at 37°C for 30 min. After precipitation with 10% trichloroacetic acid (TCA), insoluble material was collected on glass fiber filters, which were washed with 5% TCA, ethanol, and acetone and dried. Precipitable radioactivity was measured using Betafluor scintillation fluid.

Immunoprecipitation and Western blot analysis. HeLa cells on 10-cm dishes (90% confluence) were treated as described, washed with ice-cold PBS, and scraped into 0.25 ml of lysis buffer (1% [vol/vol] Nonidet P-40 [NP-40], 150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 2 mM phenylmethylsulfony fluoride [PMSF], 2 U of aprotinin per ml, 1 mM Na_3VO_4, 10 mM NaF). Lysates were centrifuged at $10,000 \times g$ for 2 min at 4°C, and the supernatants were used either for Western blot analysis or immunoprecipitation. Proteins were immunoprecipitated by mixing equivalent amounts of extract protein, determined according to Bradford (5), with 0.5 μg of antibody per mg of protein. Following incubation overnight on a rotary shaker at 4°C, 8 μg of rabbit anti-mouse Ig was added, and incubation was continued for 1 h. Immune complexes were recovered by adding 50 μl of a 50% slurry of protein A-Sepharose per sample and incubating at 4°C for 2 h. After washing extensively (150 mM NaCl, 50 mM Tris-HCl [pH 8.0], 5 mM EDTA, 0.25% [vol/vol] Triton X-100, 1 mg of ovalbumin per ml, 2 mM PMSF, 2 U of aprotinin per ml, 1 mM Na_3VO_4, 10 mM NaF), immunoprecipitated proteins were released by boiling in 50 μl of loading buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5%).

Proteins were transferred to nitrocellulose membranes using a wet transfer apparatus (Bio-Rad) in 10 mM Sodium borate at room temperature for 2 h at 45 mA. Membranes were blocked in 5% (wt/vol) nonfat dry milk (Blotto), 1% (wt/vol) bovine serum albumin (BSA), 1% (vol/vol) goat serum when using Stat1, Jak2, Jak1, or 1-3 (rabbit polyclonal) as primary Abs or with 3% BSA and 1% goat serum to probe with PY20. Primary Ab incubation was performed overnight at 4°C with the above Abs diluted 1:1,000 (1:400 for anti-1-3) in their corresponding blocking buffer. Membranes were washed in Tris-buffered saline (20 mM Tris-HCl [pH 7.4], 137 mM NaCl)–0.1% Tween 20 for 1 h, incubated with peroxidase-labeled anti-mouse Ig Ab (1:5,000) or anti-rabbit Ig Ab (1:1,000) diluted in blocking buffer for 1 h at 20°C, washed as before, and developed using the enhanced chemiluminescence detection system (Amersham) as directed by the manufacturer. Membranes were reprobed with a different Ab after stripping by incubation in 60 mM Tris-HCl (pH 6.7)–2% SDS–0.1 M β-mercaptoethanol for 45 min at 42°C.

Densitometric analysis of the blots for quantitation was done with an LKB ultroscan XL enhanced laser densitometer (LKB, Bromma, Sweden).

Immunofluorescence. Cells were grown on glass coverslips until 70% confluent. After the appropriate treatment, cells were rinsed in ice-cold Dulbecco's phosphate-buffered saline (D-PBS), fixed with cold methanol for 20 min at 20°C, and incubated with blocking buffer (1% BSA and 5% goat serum in D-PBS) overnight at 4°C. Incubation with primary Ab was performed at a 1:100 dilution for 2 h at 20°C. After rinsing thoroughly with D-PBS, rhodamine-conjugated anti-mouse Ig (1:200) was added to the coverslips, which were incubated and washed with blocking buffer. Coverslips were mounted on a drop of 90% glycerol in 0.1× PBS, overlaid with a clean coverslip, and observed in an epifluorescence microscope (Zeiss, Munich, Germany).

Expression of VH1 phosphatase. Plasmids for the expression of His-tagged wild-type VH1 (pET14b-VH1) and its catalytically inactive mutant form, $VH1^{C110S}$ (pET14b-$VH1^{C110S}$) (42), were introduced into the *Escherichia coli* host BL21(*lys*S). Expression was induced in the presence of 1 mM IPTG as described before (62), and cultures expressing VH1 were incubated at 20°C to maximize VH1 solubility. $VH1^{C110S}$ was expressed at 37°C. The proteins were purified by Ni^{2+} affinity chromatography as described in the pET system manual (Novagen Inc.).

In vitro dephosphorylation of Stat1-P with purified VH1. Lysates containing equal amounts of protein from IFN-γ-treated HeLa cells were immunoprecipitated with anti-Stat1 MAb and resolved by SDS-PAGE in replicate. Phosphor-

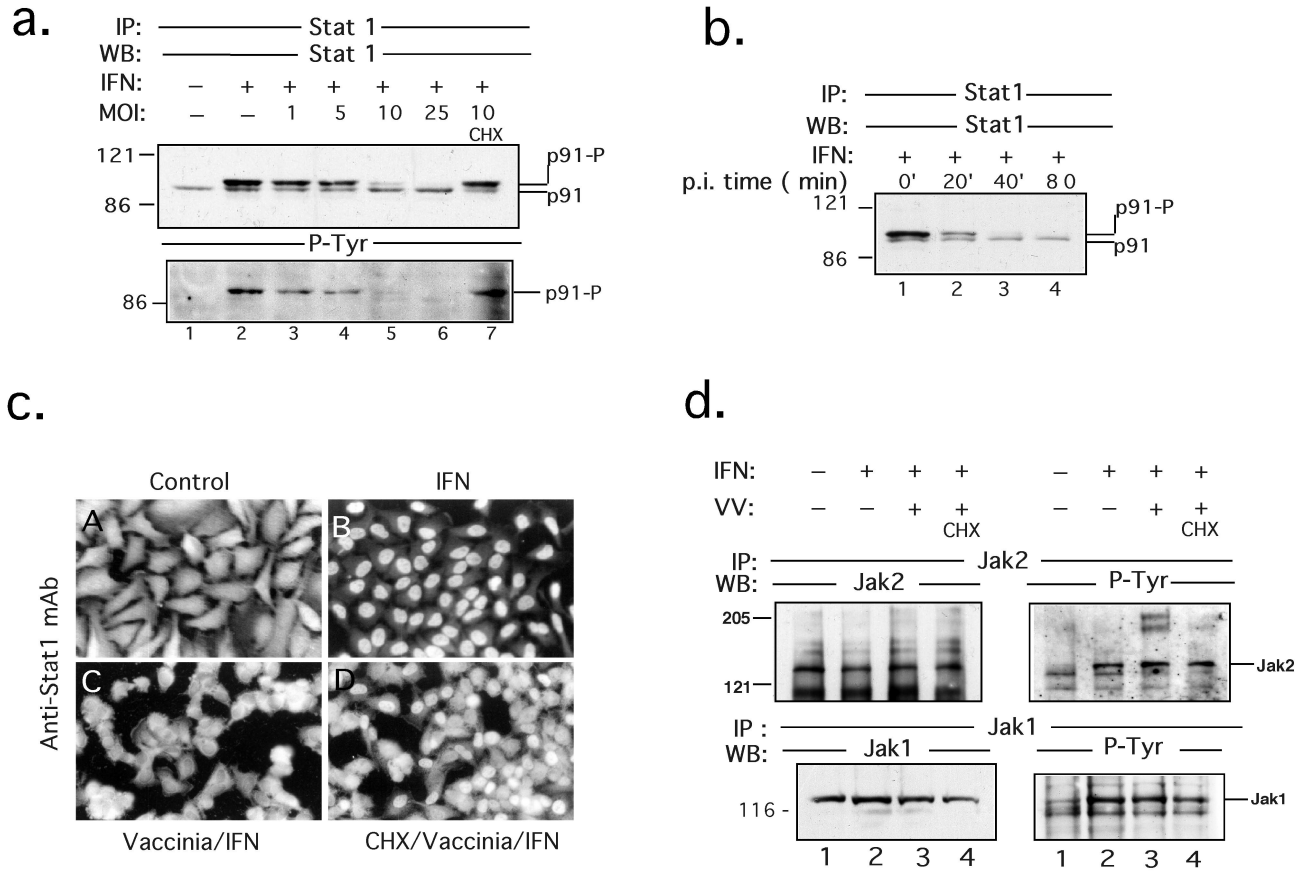

FIG. 1. VV inhibits the IFN-γ signal transduction pathway. (a) Western blot of HeLa cells infected with VV at different MOIs for 1 h before treatment with IFN-γ (200 U/ml) for 30 min. Extracts were immunoprecipitated (IP) with MAb against Stat1, and Western blots (WB) were probed with either anti-Stat1 (top) or anti-P-Tyr (bottom). Cells in lane 7 were treated with CHX (50 μg/ml) throughout. (b) Western blot of immunoprecipitates from HeLa cells infected for different times with VV (10 PFU/cell) before addition of IFN-γ for 30 min. (c) Immunofluorescent localization of Stat1 in HeLa cells. (A) Control cells; (B) cells treated with IFN-γ for 30 min; (C) cells infected with VV for 60 min before treatment with IFN-γ; (D) cells infected with VV for 60 min in the presence of CHX before treatment with IFN-γ. (d) Western blots of immunoprecipitates from HeLa cells infected with VV and treated with IFN-γ as indicated. Extracts were immunoprecipitated with either Jak2 (top) or Jak1 (bottom), and blots were developed with anti-Jak1 or -Jak2 (left) or with anti-P-Tyr (right). Sizes are shown in kilodaltons in this and all subsequent figures.

ylated Stat1 was transferred to a nitrocellulose membrane, which was then cut into separate lanes containing equal amounts of the protein and incubated in phosphatase buffer (50 mM imidazole [pH 7.5], 0.1% β-mercaptoethanol) with or without purified VH1 at a final concentration of 15 μg/ml at 22°C for 1 h. Membranes were washed in TBS-T, reprobed for P-Tyr levels, and quantitated by densitometry.

Substrate trapping using affinity support. Affinity trapping was performed as described by Todd et al. (63). Purified catalytically inactive VH1 ($VH1^{C110S}$) was coupled to Affigel 10 from Bio-Rad as described (63). As a control for nonspecific binding, the same procedure was followed without added protein. After blocking reactive sites, the gel was then transferred to a column and equilibrated with 50 mM Tris-HCl (pH 6.5)–50 mM NaCl (binding buffer). HeLa cells (90% confluent) in 150-mm plates were treated with IFN-γ (200 IU/ml) for 30 min. Cells were collected in lysis buffer (50 mM Tris-HCl [pH 6.5], 15 mM NaCl, 1% Nonidet P-40, 1 mM PMSF, 5 μg of aprotinin per ml), lysed by sonication (30 s on ice) and Dounce homogenization, and centrifuged at 15,000 rpm in an SS-34 rotor at 4°C for 10 min. Half of the supernatant was loaded on the Affigel 10 column coupled to $VH1^{C110S}$, and the other half was loaded on the Affigel 10 blank column. Columns were washed with 2 ml of binding buffer, and retained phosphoproteins were eluted with 7 ml of 0.2 mM sodium arsenate. Collected fractions were pooled, concentrated in a Speed-Vac, and analyzed for P-Tyr and Stat1 content by Western blotting.

RESULTS

VV inhibits IFN-γ-induced phosphorylation and nuclear translocation of Stat1 rapidly and in a dose-dependent manner. To determine whether viral infection directly affected the IFN-γ signal transduction pathway, we analyzed the phosphorylation levels of Stat1 after treatment with IFN-γ in cells that had been infected with VV at different multiplicities of infection (MOI). HeLa cell monolayers were synchronously infected with VV WR for 1 h and then treated with IFN-γ at 200 IU/ml for 30 min. Lysates were prepared and analyzed by Western blotting with Stat1 MAb and anti-P-Tyr MAb PY20. In control cells, IFN-γ induced phosphorylation of Stat1 within 30 min (Fig. 1a, lane 2). The phosphorylated species (p91-P) migrates more slowly than the native Stat1 (p91) (55, 57) permitting its detection by direct Western blot analysis of lysates. Stat1 phosphorylation decreased in a dose-dependent manner in cells infected with VV and was completely blocked above an MOI of 10 PFU/cell (Fig. 1a, lane 6).

This blockage of Stat1 phosphorylation during VV infection was also observed in other cell lines, such as A431 and 293 cells. The IFN-γ signal transduction pathway can be activated in the presence of protein synthesis inhibitors because the components of the signaling cascade are latent cytoplasmic transcription factors (56) (data not shown). When protein synthesis was inhibited by adding cycloheximide (CHX, 50 μg/ml) to the medium of VV-infected cells, the band corresponding to p91-P was as strong as in control samples (Fig. 1a, lane 7). Similar results were obtained with other protein synthesis inhibitors, emetine, anisomycin, and puromycin (data not shown). Time course studies, in which infection was permitted for different times before IFN-γ treatment, showed that Stat1 phosphorylation is reduced as early as 20 min after infection and almost completely inhibited when cells are infected with VV for 40 min or more (Fig. 1b). The results suggested that a virion component is responsible for the inhibition of Stat-1 phosphorylation.

Activation of Stat1 requires that it bind to the IFN-γR and be phosphorylated at the membrane before translocation to the nucleus. To determine if Stat1 nuclear translocation was blocked during VV infection, HeLa cells were grown on coverslips and then infected with VV at an MOI of 25 for 1 h. IFN-γ was then added to the cells, and after incubation for 30 min, localization of Stat1 was performed by indirect immunofluorescence. Addition of IFN-γ caused virtually all fluorescent signal, dispersed over the cytoplasm in control cells, to accumulate in the nucleus (Fig. 1c, A and B). VV-infected cells showed some rounding as a consequence of the VV-induced cytopathic effect, which is associated with viral protein synthesis (3). Nuclei of infected cells stained very weakly after addition of IFN-γ, indicating that translocation of Stat1 to the nucleus was inhibited (Fig. 1c, C). Inhibition of protein synthesis with CHX allowed nuclear translocation of Stat1 to take place during infection with VV (Fig. 1c, D), suggesting, as seen previously for the p91-P levels, that host and/or viral protein synthesis was required for VV to exert its inhibitory effect on the IFN-γ signaling pathway.

The ability of other viruses such as Influenza virus (orthomyxovirus), vesicular stomatitis virus (VSV) (rhabdovirus), and mengovirus (picornavirus) to modulate Stat1 phosphorylation was analyzed. None of these viruses affected the phosphorylation levels or nuclear translocation of Stat1 after the infected cells were treated with IFN-γ. However, the highly modifed VV Ankara (MVA) had the same effect on Stat1 activation as the WR strain (data not shown). The MVA strain has multiple deletions and substitutions in its genome that alter, among others, viral anti-host defense proteins, including the VV IFN-γR antagonist.

Phosphorylation of other signaling molecules in the IFN-γ pathway is not affected by VV. We next analyzed other components of the phosphorylation cascade during IFN-γ signaling to see if they too were affected by VV. Jak1 and Jak2 associate with the IFN-γR α and β chains, respectively, and are rapidly phosphorylated upon IFN binding (54). The IFN-γRα chain is also phosphorylated upon ligand binding and constitutes a docking site for the binding of Stat1 (22). HeLa cells were treated for 20 min with IFN-γ (500 IU/ml) after being infected with VV for 30 min, and lysates were immunoprecipitated with anti-Jak2, anti-Jak1, or anti-IFN-γRα chain MAbs. Jak1 and Jak2 proteins were detected on Western blots of the immunoprecipitates with the corresponding MAbs, and P-Tyr was detected with anti-P-Tyr (PY20) MAb. Phosphorylation of Jak2 and Jak1 was only observed after treatment with IFN-γ and was unaffected by VV infection with or without CHX (Fig. 1d). A similar result was obtained for IFN-γRα chain (data not shown). Analysis of the same extracts for Stat1 showed that VV infection had blocked phosphorylation of Stat1 (not shown). These results indicate that the inhibition seen for Stat1 phosphorylation is selective and that upstream phosphorylation is not affected during VV infection.

VV blocks induction of IFN-γ-responsive genes. We reasoned that VV-mediated blockage of Stat1 phosphorylation and nuclear translocation would inhibit the accumulation of IFN-induced mRNAs. In order to test this point, we performed Northern blot analyses to determine the mRNA levels of genes whose transcription is induced by IFN-γ. To facilitate the interpretation of the results, we avoided the inhibitory effect of VV on cellular mRNA levels (49, 52) by blocking VV protein synthesis with adenosine N_1-oxide (ANO). It has been shown previously that in BHK cells treated with ANO, early viral mRNAs are produced but VV early polypeptides are not synthesized due to selective incorporation of the adenosine analogue into viral mRNA (33). To ensure that ANO inhibits synthesis of VV proteins in HeLa cells in the same way previously reported for BHK cells, Western blot analysis of an early protein, I3, was performed with cells treated with various doses of ANO. After a 5-h treatment with the drug, cells were infected with VV (10 PFU/cell), and lysates were prepared at 1 and 2 h p.i. Western analysis showed the presence of I3, a protein that is not encapsidated (53), in cells as early as 1 h p.i. and strongly at 2 h p.i. (Fig. 2a). In cells treated with as little as 5 μg of ANO per ml, I3 was undetectable at both 1 and 2 h p.i. (Fig. 2a), showing that viral early protein synthesis was blocked by the drug. Experiments in which HeLa cells treated for 5 to 10 h with ANO (20 μg/ml) were labeled with [^{35}S]methionine for 30 min showed that the drug had no effect qualitatively (SDS-PAGE analysis) or quantitatively (TCA-insoluble counts) on host protein synthesis (data not shown). As expected, ANO prevented the appearance of virus-specific proteins in [^{35}S]methionine-labeled, VV-infected cells, showing that this drug selectively inhibits viral protein synthesis in HeLa cells.

HeLa cells were grown to 75% confluency and incubated with or without ANO at 20 μg/ml for 5 h. VV was then adsorbed to the cells (MOI of 10), and at 30 min p.i., the inoculum was removed and IFN-γ (500 IU/ml) was added for 2 or 4 h, as indicated. Northern blot analysis of total mRNA is shown in Fig. 2b. In control cells, both Stat1 (50) and the guanylate binding protein (GBP) (11) mRNA levels increased substantially after exposure to IFN-γ for 2 h and were further augmented at 4 h (Fig. 2b, lanes 1 to 3). Under the same conditions, actin mRNA levels remained constant. During VV infection, the levels of actin mRNA were strongly reduced (lanes 4 to 6) unless ANO was present (lanes 9 and 10). No induction of GBP or Stat1 mRNAs was detected in cells infected with VV prior to IFN-γ treatment, while a strong reduction in actin mRNA steady-state levels was observed (lanes 7 and 8). Preincubation with ANO alone did not alter cellular mRNA levels (lanes 9 and 10) and had no effect on the induction of GBP or Stat1 mRNA by IFN-γ (not shown). When cells

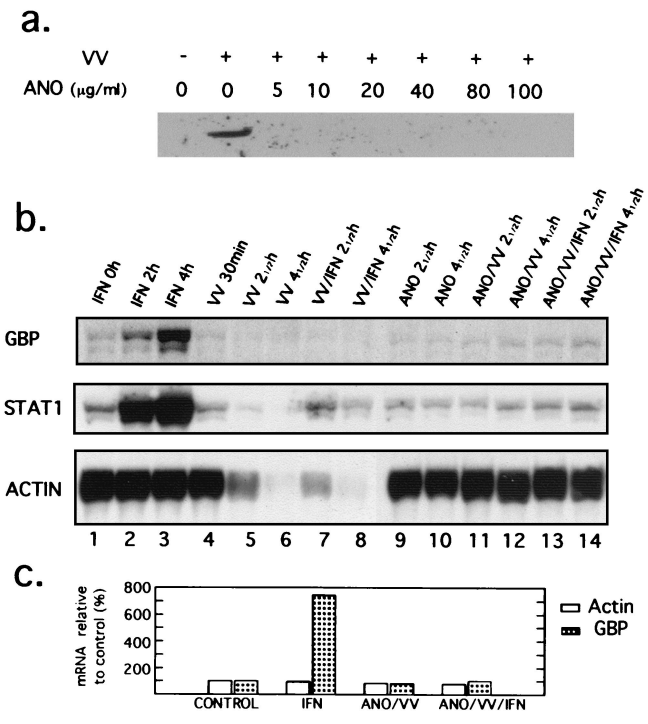

FIG. 2. VV blocks induction of mRNA transcription by IFN-γ. (a) ANO blocks early viral protein synthesis in HeLa cells. Cells were treated with ANO for 5 h at the concentrations indicated, VV was adsorbed at 4°C (10 PFU/cell), and after addition of growth medium, infection was allowed to proceed for 2 h in the presence of ANO at the concentration shown. Lysates were analyzed by Western blotting with antiserum against early protein I3. (b) Northern blot analysis of total cell RNA from control and VV-infected HeLa cells. Cells were treated with ANO (20 μg/ml) for 5 h or not and then mock infected or infected with VV (10 PFU/cell). After 30 min of infection, cells were treated with IFN-γ (200 U/ml) in the absence or presence of ANO for 0, 2, or 4 h. Times shown indicate time p.i. RNA was isolated and analyzed by Northern blotting using probes for GBP, Stat1, and actin. (c) Quantitation of densitometric signals for selected treatments (4.5 h). Treatment with ANO had no effect on induction by IFN-γ (not shown).

were infected with VV in the presence of ANO, treatment with IFN-γ failed to cause induction of Stat1 and GBP mRNAs even though the basal levels of housekeeping genes were unaffected (lanes 13 and 14). Quantitation of the data obtained for cells in the presence of ANO is shown in Fig. 2c and indicate that the sevenfold induction of GBP levels was completely abolished in VV-infected cells when viral cytopathic effects were blocked by treatment with ANO.

Viral protein synthesis not required for VV to interfere with IFN-γ signaling pathway. As described earlier, inhibiting protein synthesis with CHX blocked the effect of VV on IFN-γ-mediated Stat1 phosphorylation. To determine whether viral or host protein synthesis was required, we selectively blocked viral translation by use of the VV-specific protein synthesis inhibitor ANO (see above). Levels of phosphorylated Stat1 after treatment with IFN-γ were analyzed by immunoprecipitation in cells infected with VV with or without ANO. As shown in Fig. 3a, ANO alone had no effect on IFN-γ-induced Stat1 activation (lane 9) or on the ability of VV to block Stat1 phosphorylation (lane 8). Thus, the synthesis of viral proteins is apparently not required for VV to block Stat1 phosphorylation.

Synthesis of VV proteins is also blocked when cells are infected with UV-irradiated virus (4). In vivo studies demonstrated that increases in the UV dose caused a progressive reduction in the ability of VV to synthesize RNA (4). Infection of HeLa cells with VV irradiated with low doses of UV (up to 9,000 erg/mm^2) in the absence of CHX (Fig. 3a and b, lanes 4 and 5) caused a reduction in p91-P levels identical to that seen with untreated virus (Fig. 3a and b, lane 3). CHX reversed this effect for both untreated virus and VV irradiated with UV from 1,300 to 5,100 erg/mm^2 (Fig. 3a, lanes 6 and 7). Western blot analysis showed that synthesis of the early VV protein I3 was strongly inhbited when virus was exposed to a UV dose of 2,400 erg/mm^2 and undetectable in virus irradiated at 5,100 or 9,000 erg/mm^2 (Fig. 3c). When total protein synthesis was blocked with CHX, the reduction in p91-P levels was very weak (Fig. 3a, lanes 6 and 7). These results suggest that a labile or de novo-synthesized cellular protein is required for VV to exert its inhibitory effect on IFN-γ signal transduction.

Viral uncoating required for VV blockage of Stat1 phosphorylation. The rapid effect of VV on IFN-γ-induced Stat1 phosphorylation, the dose dependency, and the lack of a need for viral protein synthesis suggested that a virion component was responsible. In order to affect Stat1 phosphorylation, such a component must likely be delivered to the cytoplasm. This hypothesis would explain the need for cellular protein synthesis, which has previously been shown to be required for virus uncoating (32).

After binding and internalization, VV virions undergo uncoating in two steps (31, 32). The first uncoating step occurs shortly after penetration, permitting expression of immediate-early genes. A second uncoating step permits replication of viral DNA. VV uncoating requires protein synthesis and, while unaffected by low-dose UV, is blocked by high (>9,200 erg/mm^2) doses of UV irradiation (4). To determine whether the ability of VV to inhibit IFN-γ signaling was dependent on uncoating, HeLa cells were infected with VV that had been irradiated with UV at low or high doses, and the effect on Stat1 phosphorylation was examined. As shown earlier, lowdose UV irradiation had no effect on the ability of virus to block IFN-γ-induced Stat-1 phosphorylation (Fig. 3b, lanes 2 to 5). However, VV irradiated at doses over 9,000 erg/mm^2 were unable to block IFN-γ-induced phosphorylation of Stat1 (Fig. 3b, lane 6), as was also seen for untreated virus in the presence of CHX (lane 7). The same results were obtained in three independent experiments.

To determine whether there was a correlation between the ability to block Stat-1 phosphorylation and virion uncoating, we also monitored the sensitivity of viral DNA to DNase (49). Cells were infected with UV-irradiated [^{3}H]VV (low dose, 3,000 erg/mm^2; high dose, 27,000 erg/mm^2) or with untreated [^{3}H]VV in the presence of CHX. Untreated virus is uncoated during infection, leading to an average sensitivity to DNase treatment of 42% (Fig. 3d). Low-dose UV irradiation did not significantly affect the uncoating of VV. However, virus exposed to doses of UV of 27,000 erg/mm^2 exhibited 92.2% resistance to DNase treatment, indicating that uncoating of the virions was blocked. Similarly, when infection was carried out in the presence of CHX, more than 98% of DNA remained

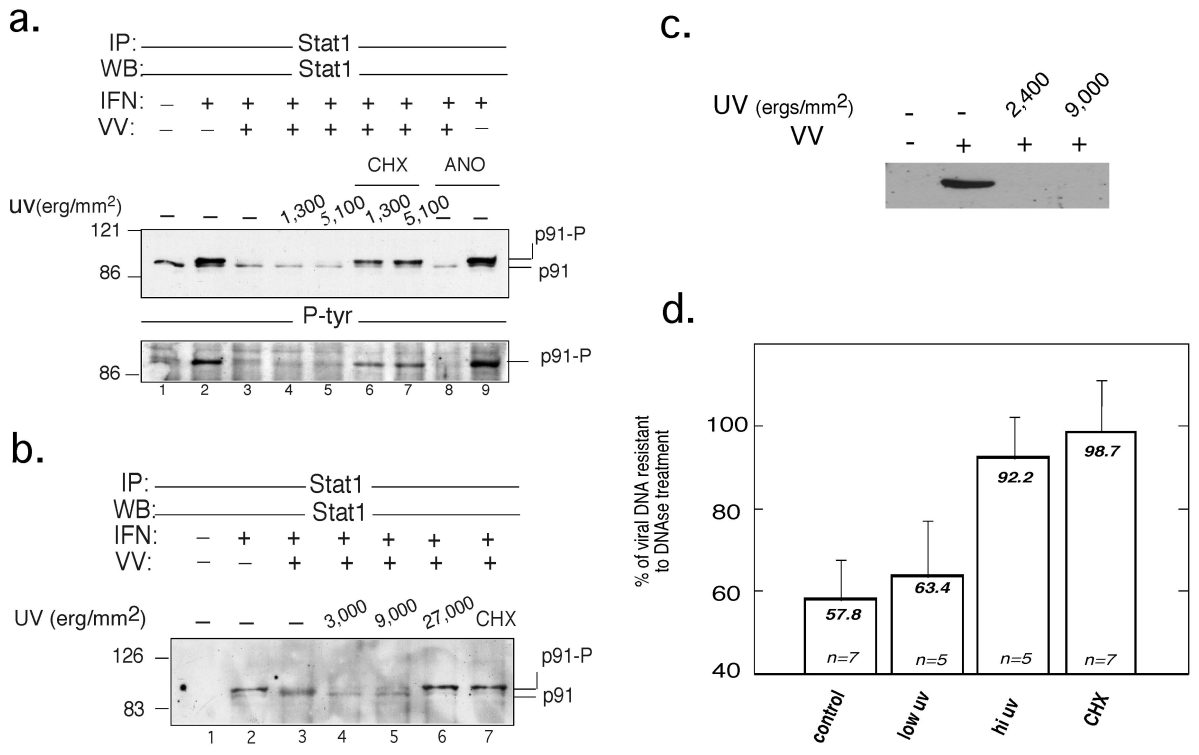

FIG. 3. CHX and UV irradiation of virus prevent the block of IFN-γ signal transduction. (a) Western blot (WB) analysis of Stat1 phosphorylation in HeLa cells treated with IFN-γ after infection with VV irradiated with various doses of UV without (lanes 4 and 5) or with (lanes 6 and 7) CHX (50 μg/ml) or with ANO (lanes 8 and 9). (b) Western blot analysis of Stat1 phosphorylation in HeLa cells treated with IFN-γ after infection with untreated VV (lanes 3 and 7) or VV irradiated with a low (lanes 4 and 5) or high (lane 6) dose of UV. (c) Western blot analysis of early protein I3 production in cells infected (10 PFU/cell) for 2 h with untreated VV (−) or VV irradiated with UV at 2,400 or 9,000 erg/mm^2. (d) Uncoating of VV analyzed by DNase sensitivity of internalized, [^{3}H-]thymidine-labeled virions. Cells were harvested at 2 h p.i., lysates were treated with DNase, and acid-insoluble material was determined in a liquid scintillation counter.

TCA precipitable. These results are in accord with the idea that uncoating of virions is required for VV to block IFN-γ signaling.

Dephosphorylation kinetics of activated Stat1 in VV-infected cells. Stat1 phosphorylation can be detected as early 15 min after binding of IFN-γ and remains detectable for more than 2 h (57; unpublished observations). Termination of the response seems to be caused by dephosphorylation (25), although ubiquitination and protein degradation may also play a role (36). In order to determine whether VV infection causes dephosphorylation of existing, activated Stat1, we examined p91-P levels in cells treated with IFN-γ and then infected with VV in the presence or absence of the viral protein synthesis inhibitor ANO. Cells were collected at different times p.i., and Stat1 phosphorylation was determined by P-Tyr blotting and quantitated by densitometry (Fig. 4). Dephosphorylation rates relative to the control (0 min p.i.) are shown in Fig. 4b. More than 70% of phosphorylated Stat1 in uninfected cells remained after 120 min (Fig. 4a, lane 12). Within 40 min of infection with VV, there was a significant decrease in p91-P levels compared to noninfected cells. The apparent $t_{1/2}$ of phosphorylated Stat1 decreased from ~80 min in uninfected cells to ~25 min in VV-infected cells (Fig. 4b). This enhancement of the rate of dephosphorylation of Stat1 did not require viral protein synthesis, since cells treated with ANO showed a similar loss of p91-P. Differences between control and infected cells were maximal at 60 min p.i., when only 5% of the initial p91-P remained in infected cells, compared to 70% in control cells (Fig. 4b). In contrast, infection with VV had no effect on the steady-state level of Stat1 whether cells were treated with IFN-γ or not (Fig. 4c), eliminating the possibility that VV acts by causing degradation of Stat1. These results indicated an increased dephosphorylation activity associated with the early steps of VV infection. Thus, the effect of VV on the IFN-γ signaling pathway is most likely due to a dephosphorylation event.

Effect of phosphatase inhibitors on Stat1 phosphorylation in cells infected with VV. Our data strongly suggested that a virion component was responsible for the impairment of IFN-γ signaling. Given the results described in the previous section, this component is likely to be a virion-associated phosphatase. VV ORF H1 encodes a dual-specificity, soluble phosphatase VH1 (23) that is packaged in the viral particles at about 200 molecules per virion. This enzyme is essential for viral transcription in vitro and in vivo (42). Sodium orthovanadate (Na_3VO_4) specifically blocks its activity, while OA does not (23). To determine whether the viral phosphatase was the virion component that caused a decrease in Stat1 phosphorylation, we tested the effect of Na_3VO_4 and OA on responses to IFN-γ (Fig. 5a).

Phosphorylation of Stat1 was not detected in cells incubated with Na_3VO_4 or OA alone (Fig. 5a, lanes 1 and 2). Addition of

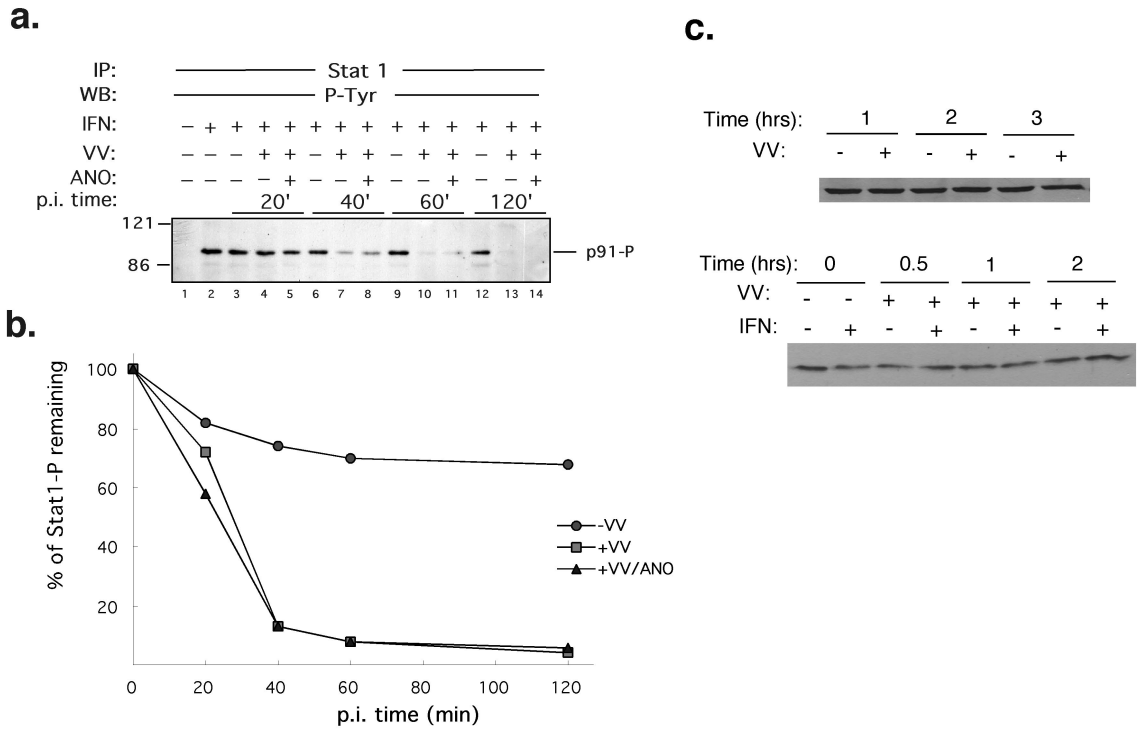

FIG. 4. Enhanced dephosporylation of Stat1-P in cells treated with IFN-γ and then infected with VV. (a) Western blot (WB) analysis of Stat1 phosphorylation in HeLa cells treated with IFN-γ for 30 min and then infected with VV for various times in the absence or presence of ANO. After treatment with IFN-γ (200 IU/ml) for 30 min, cells were washed to remove excess IFN and then mock infected (−VV) or infected with VV at 10 PFU/cell (+VV). The cells were harvested at various times after infection, and equal amounts of cell protein were immunoprecipitated (IP) with an antibody against Stat1 followed by Western blotting with anti-P-Tyr. The migration position of p91-P is shown at the right. Control uninfected cells, untreated (lane 1) or treated with IFN-γ (lane 2) and harvested at time zero are shown for comparison. A parallel batch of cells was processed identically except they were treated with ANO (20 μg/ml) for 5 h and maintained in the presence of this drug throughout (+VV/ANO). (b) Quantitation of data shown in panel a as a percentage of levels in uninfected cells after treatment with IFN-γ for 30 min. (c) Western blot analysis of Stat1 levels in HeLa cells infected with VV. (Upper panel) Cells were mock infected or infected with VV (10 PFU/cell) and cultured for the times indicated. (Lower panel): Mock-infected or VV-infected cells (10 PFU/cell) were incubated for the times shown before addition of IFN-γ (200 U/ml) for 30 min. Extracts were analyzed by SDS–10% PAGE so that the phosphorylated and unphosphorylated proteins were not separated, allowing total Stat1 levels to be discerned.

IFN-γ in the presence of phosphatase inhibitors induced the same levels of p91-P as with IFN-γ alone (compare lanes 5 and 6 with lane 3). Stat1 phosphorylation was inhibited in VV-infected cells (lane 4), but addition of Na_3VO_4 overcame the ability of VV to block Stat1 phosphorylation (lane 7). In contrast, OA had no effect on the ability of VV to inhibit IFN-γ signal transduction (lane 8). Similar results were obtained when the effects of Na_3VO_4 and OA on Stat1 nuclear localization were examined (data not shown).

VV deficient in VH1 is unable to prevent Stat1 phosphorylation. To provide further evidence that VH1 is responsible for blocking the IFN-γ signaling pathway, we used VH1-deficient VV mutants. VH1 is essential for VV replication, since attempts to generate null mutants for the phosphatase were unsuccessful (42). Using an inducible mutant (vindH1) in which H1 expression is under the control of the *lac* repressor-operator system, Liu et al. showed that VH1 is required for the infectivity and transcriptional competence of nascent virions (42). Virions containing low levels of VH1 are defective in the early steps of infection, including early transcription and hence viral protein synthesis (42). Virions generated in the presence ($H1^+$) or absence ($H1^-$) of the Lac inducer IPTG contained 16.7 and 2.4%, respectively, of the level of VH1 encapsidated in wild-type (wt) virions (42). We infected HeLa cells with wt, $H1^-$, and $H1^+$ virions, applying the same number of particles per cell (500 particles/cell; equivalent to 10 PFU of wt virus/cell). Virus entry is not compromised in these mutants (42). Cells were then washed, and infection was continued for 30 min prior to the addition of IFN-γ for an additional 30 min. Western blot analysis of the cell lysates (Fig. 5b) showed that neither $H1^+$ nor $H1^-$ virions were able to inhibit Stat1 phosphorylation to the extent that wt virus did. Moreover, the levels of p91-P after infection with $H1^-$ and $H1^+$ viruses showed an inverse correlation with the amount of VH1 included in the virions. These results strongly reinforce the hypothesis that VH1 phosphatase is required to block the IFN-γ signal pathway in the early phase of VV infection.

Phosphorylated Stat1 is a substrate for VH1 in vitro. To examine the possibility that phosphorylated Stat1 is a substrate for VH1, we tested the ability of purified, recombinant VH1 to dephosphorylate p91-P. HeLa cells were treated with IFN-γ, and cell lysates were immunoprecipitated with anti-Stat1. Equal amounts of the recovered proteins were analyzed by Western blotting with anti-P-Tyr Ab (Fig. 6a, upper panel).

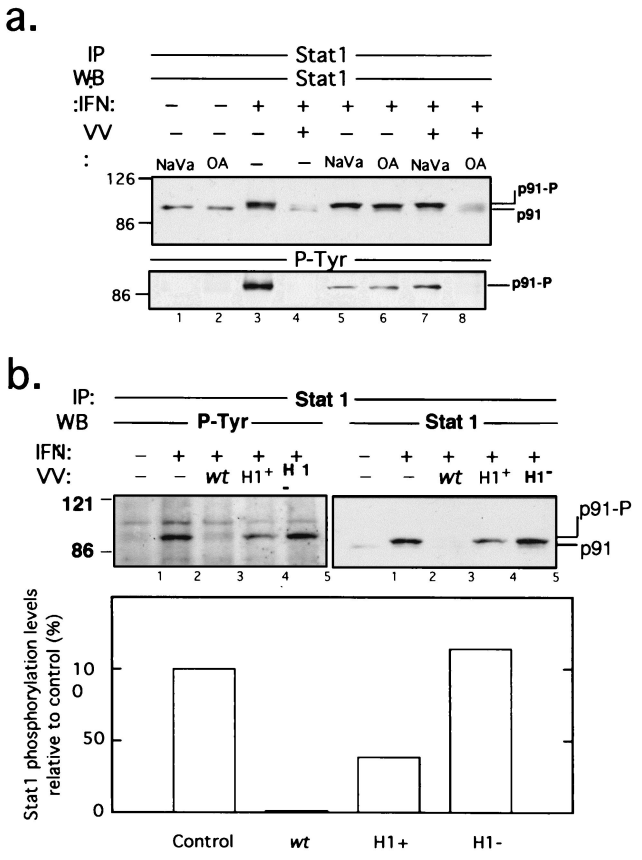

FIG. 5. VV VH1 phosphatase activity correlates with the virus-induced block in IFN-γ signal transduction. (a) Western blot (WB) analysis showing effect of phosphatase inhibitors sodium vanadate (NaVa) and OA on Stat1 phosphorylation. HeLa cells were incubated with $NaVO_4$ (lanes 1, 5, and 7) or OA (lanes 2, 6, and 8) and either infected with VV (lanes 4, 7, and 8) or left uninfected (lanes 1, 2, 3, 5, and 6). After treatment with IFN-γ for 30 min, extracts were prepared, and immunoprecipitates (IP) were analyzed by SDS-PAGE and transferred to a membrane. After being probed for Stat1 (top panel), the blot was stripped and reprobed for P-Tyr (lower panel). (b) Western blot analysis of Stat1 phosphorylation in cells infected with VH1 variant virus. HeLa cells were infected with equivalent numbers of particles (500 per cell) of wt WR strain or VH1 mutant strain produced in the presence ($H1^+$) or absence ($H1^-$) of IPTG. After treatment with IFN-γ (200 U/ml) for 30 min, extracts were immunoprecipitated, and blots were probed for P-Tyr (left panel) or Stat1 (right panel). Quantitation of the P-Tyr blot is shown below.

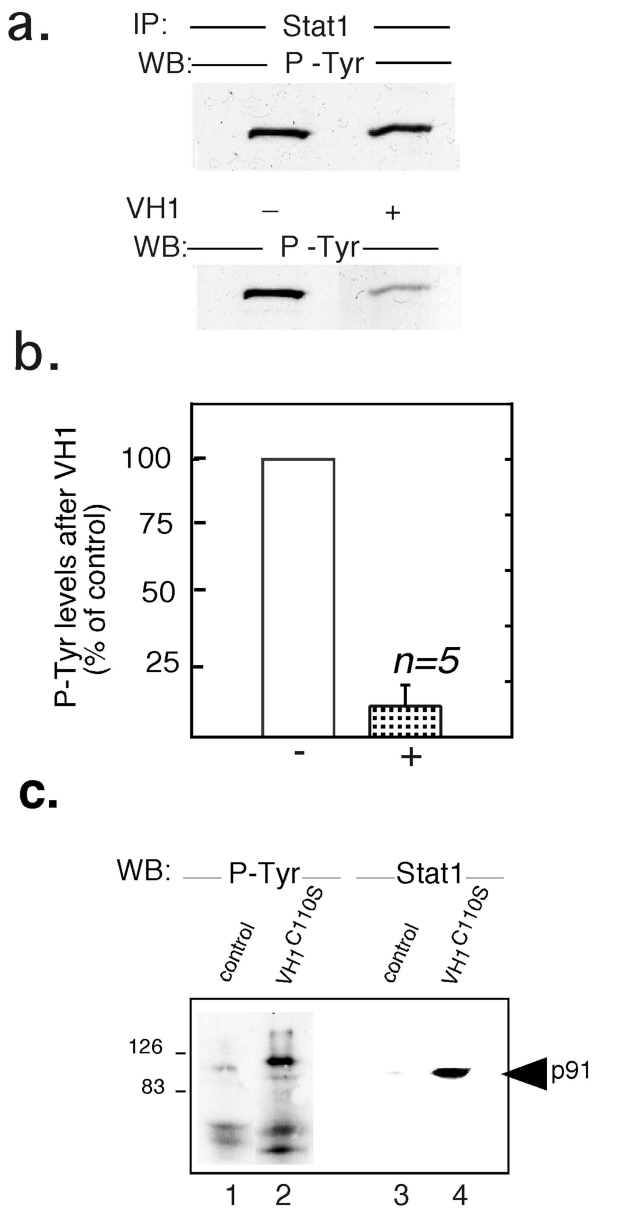

FIG. 6. Phosphorylated Stat1 serves as a substrate for VH1 phosphatase in vitro. (a) Sensitivity of denatured p91-P to VH1 shown by incubating Western blots (WB) containing immunoprecipitated (IP) p91-P from IFN-γ-treated HeLa cells (upper panels) without (bottom left) or with (bottom right) recombinant VH1 phosphatase, followed by reprobing with anti-P-Tyr MAb. (b) Quantitation of the data from panel a (mean ± standard deviation of five experiments). (c) Substrate trapping identifies p91-P as a substrate for VH1 phosphatase. Extracts of IFN-γ-treated HeLa cells were applied to uncoupled (control) or $VH1^{C110S}$ mutant, recombinant VH1-coupled ($VH1^{C110S}$) Affigel columns. After washing, bound proteins were eluted with 0.2 mM sodium arsenate and analyzed by Western blotting (WB) for P-Tyr (left panel) and, after stripping, reprobed for Stat1 (right panel).

After detection, the membrane was stripped and cut in half. The left lane was incubated with buffer alone, and the right lane was incubated with buffer containing recombinant VH1 at a final concentration of 15 μg/ml. The membranes were then reprobed for P-Tyr levels. We consistently observed a strong reduction (>80%) in the p91-P levels in samples incubated with VH1 compared to the control. Densitometric quantitation of five independent experiments is shown in Fig. 6b. Together, these data indicated that, at least in vitro, Stat1 is a bona fide substrate of VH1.

Mutations in the catalytic site of phosphatases have been used to generate stable enzyme-substrate complexes that permit the identification of putative substrates of these enzymes (21). We used catalytically inactive VH1 ($VH1^{C110S}$) coupled to Affigel−10. Equal amounts of protein from extracts of IFN-γ-treated HeLa cells were loaded on the $VH1^{C110S}$ and control columns. Bound proteins (Fig. 6b) were eluted with the competitive inhibitor sodium arsenate, which displaces only phosphorylated proteins associated with $VH1^{C110S}$. Eluates from

the $VH1^{C110S}$ column (Fig. 6c, lane 2) contained several proteins containing P-Tyr that were not retained or bound only weakly to the control column (Fig. 6b, lane 1). One of the phosphorylated proteins specifically retained was identified as Stat1 by reprobing the membrane with anti-Stat1 Ab (Fig. 6c, lanes 3 and 4). As predicted from previous studies (23), VH1 can interact with a broad range of substrate proteins containing P-Tyr. Our results suggest that Stat1 is among these proteins.

DISCUSSION

IFN-γ is a key cytokine involved in protection against viral infection. IFN produced in response to virus infection induces IFN-responsive cells to create an antiviral state that efficiently prevents the spread of the virus. Knockout mice deficient for IFN-γR (26, 48) show increased susceptibility to infection by VV but not to VSV or Semliki Forest virus. Viruses have evolved a series of strategies to counteract this host defense mechanism. In particular, VV has several genes encoding proteins that interfere with responses to IFNs. The products of VV genes E3L and K3L inhibit the activation and/or activity of PKR (6, 8). Moreover, the VV analog of the IFN-γR encoded by gene B8R competes with and blocks the binding of IFN-γ to its natural receptor (46, 59). We demonstrate here that VV can block IFN-γ responses by another mechanism, inhibiting the IFN-γ signal transduction pathway. The data presented here strongly support a role for the virion-associated VH1 phosphatase in this effect.

VV blocks IFN-γ signal transduction. Stat1 phosphorylation and nuclear translocation were found to be blocked only during infection with poxviruses (VV strains WR and MVA), while infection with viruses as diverse as an orthomyxovirus (influenza virus), picornavirus (mengovirus), and rhabdovirus (VSV) did not affect the pathway. This specificity eliminates the possibility that nonspecific consequences of viral infection (e.g., virus entry, viral mRNA transcription, or host shut-off) were responsible for the effect. Moreover, since the MVA variant also blocked Stat1 phosphorylation, we can rule out the possibility that the response is due to neutralization of IFN-γ by the IFN-γR homologue, which is deleted in this strain (2). Further evidence that the effect is not due to incapacitation of IFN-γ is provided by the fact that while Stat1 phosphorylation is blocked by VV, the phosphorylation uf Jak 1 and 2 and the IFN-γR still occurs in response to IFN-γ in VV-infected cells.

IFN-γ signaling leads to the enhancement of several mRNA species, and some of them have been directly implicated in the antiviral responses induced by this cytokine (61). Under conditions in which viral protein synthesis is inhibited while host transcript levels are essentially unchanged, treatment with IFN-γ failed to induce the accumulation of GBP or Stat1 mRNA. Since this effect does not appear to be due to a reduction in mRNA half-lives by VV, it is most likely a consequence of the block of the IFN-γ signaling pathway during early VV infection. The IFN system is able to impair different steps of viral replication, and it is not surprising that VV has evolved various apparently redundant mechanisms to counteract these effects. The lack of mRNA stimulation by IFN-γ in virus-infected cells observed in this study demonstrates an additional viral strategy acting at a step prior to those interfering with PKR or oligo-2,5A synthetase functions. Blocking the signal transduction pathway is a strategically effective way of preventing IFN from activating an antiviral response in cells that are already infected.

The activation of Stat1 after ligand binding is preceded by a series of kinase activities at the cell membrane. None of the kinase responses examined (Jak1, Jak2, and IFN-γRα) were affected during VV infection, indicating that the reduced level of phosphorylated Stat1 was specific and not due to a general inhibitory effect of VV on protein tyrosine phosphorylation. This was true for both IFN-γ and epidermal growth factor (EGF) (data not shown) stimulation of infected cells.

Blockage of the IFN-γ signaling pathway requires a virion component. Inhibition of Stat1 phosphorylation occurs very rapidly after VV infection, even in the presence of ANO, suggesting that translation of early, intermediate, or late VV mRNAs is not needed. Complete inhibition of Stat1 phosphorylation occurred at MOIs above 10, at which level more than 90% of the cells are infected. Other cell lines infected with VV showed the same response. In the epitheloid carcinoma cell line A431, complete inhibition of Stat1 phosphorylation after IFN-γ treatment was achieved at 10PFU/cell, as also was phosphorylation of Stat1 induced by EGF (data not shown).

Although IFN-γ induced translocation of Stat-1 is unaffected by protein synthesis inhibitors for up to 4 h, the presence of CHX in the incubation medium during VV infection blocked the effect of VV on Stat1 phosphorylation levels and nuclear translocation. This suggests a need for ongoing protein synthesis, a virus-induced cellular cofactor, or a newly synthesized viral protein. We excluded a need for viral protein synthesis by using a specific inhibitor of viral protein synthesis (ANO) or by infection with low-dose UV-irradiated virus. As we have shown, synthesis of a characteristic early protein, I3, is blocked under each of these conditions, indicating that both ANO and low-dose UV irradiation prevent the synthesis of early viral proteins in general. The effect of CHX therefore seems unlikely to be due to a blockage of viral protein synthesis. Several other protein synthesis inhibitors (emetine, anisomycin, and puromycin) had the same effect, and the dose response for each correlated with that needed to block protein synthesis. It is thus unlikely that the response to CHX can be ascribed to secondary effects rather than to inhibition of protein synthesis.

Since our data suggest that a virion component is responsible for the effect of VV on Stat1 activation, a likely explanation for the effect of protein synthesis inhibitors is the dependence of viral uncoating on host protein synthesis (32). Two approaches have been taken to analyze viral uncoating of [^{3}H]thymidine-labeled VV. One is based on the production of TCA-precipitable material with characteristic sedimentation rates (whole virus, cores, and viral DNA) as the infection progresses (4, 32, 43, 55). Cores accumulate in the presence of actinomycin D (43) or when the inoculum is treated with UV intensities over 9,200 erg/mm^2 (4). The other approach relies on the increased DNase sensitivity of the viral genome as uncoating progresses; whole virus and cores are DNase resistant until the second uncoating step takes place, rendering 50% of the labeled DNA acid soluble. Both approaches show that secondary uncoating requires both RNA and protein synthesis. Our data confirm that secondary uncoating is blocked

by high but not low doses of UV exposure. Similarly, low UV doses had no effect on the inhibition of Stat1 phosphorylation, while high UV doses prevented this block. This finding strengthens the idea that a viral core component is responsible for the inhibitory effect and that uncoating of virions is needed to release this viral factor into the cytoplasm. High-dose UV irradiation increases the sedimentation rate of viral DNA, which correlates with the cross-linking of proteins to viral DNA (4). It is thus possible that the normal phosphorylation levels detected in IFN-γ-treated cells infected with high-dose UV-irradiated VV result from the sequestering of an inhibitory activity present within the uncoated cores. Although it is widely accepted that secondary uncoating requires protein synthesis, the factor responsible for viral uncoating (49) remains to be identified. Thus, it is likely that the normal phosphorylation levels obtained in the presence of protein synthesis inhibitors such as CHX are due to the inhibition of the secondary uncoating step needed for release of a virion component into the cytoplasm. The importance of the initial steps in the VV infectious cycle is underscored by studies showing that pretreatment of cells with IFN-α/β blocks uncoating of VV in chicken embryo fibroblasts, leading to an accumulation of cores (44), as seen when infection is carried out in the presence of CHX.

Virion component responsible for the effect is a phosphatase (VH1). Stat1 protein has a half-life of about 24 h (39). During its activation after IFN-γ treatment, it can undergo several cycles of phosphorylation and dephosphorylation, and the estimated half-life of an activated p91-P molecule is 15 min (25). The fact that VV blocks Stat1 phosphorylation prompted us to compare the decay of Stat1 activation in uninfected cells and cells infected with VV in which a pool of active Stat1 molecules had been accumulated by IFN-γ stimulation. The strong reduction in p91-P steady-state half-life after infection with VV suggested that the lack of phosphorylation detected in previous experiments was indeed the result of a virion-associated phosphatase activity. Stat1 phosphorylation levels were greatly reduced after 40 min of virus infection in IFN-γ-treated cells ($t_{1/2}$ of p91-P was reduced to 30% of control values in uninfected cells), even when viral protein synthesis was inhibited. While the levels of phosphorylated Stat1 declined markedly during VV infection, there was no alteration in the steady-state level of p91 whether the cells were treated with IFN-γ or not. This rules out the possibility that VV promotes degradation of Stat1 by proteolysis, as has been observed with another class of virus (20). Our data thus support a role for a viral phosphatase in blocking signal transduction by IFN-γ.

The importance of protein tyrosine phosphatases in IFN-γ-stimulated gene expression has been documented by studies in which Stat1 phosphorylation was induced and/or prolonged in the presence of phosphatase inhibitors (10, 28). Phosphatases may play a role by turning off the Stat1 signal or by repressing the pathway that leads to its phosphorylation (13, 27, 38). Since no inhibition of Jak1, Jak2, or IFN-γR phosphorylation was observed, it is likely that VV acts to dephosphorylate p91-P specifically.

Analysis of virus-associated phosphatase activity (VH1) after detergent permeabilization of VV shows that part of VH1 remains core associated and part is released to the supernatant fluid (42). VH1 was a good candidate for the mediator of VV-induced blockage of IFN-γ signaling. We took advantage of its differential sensitivity to phosphatase inhibitors to implicate VH1 in Stat1 dephosphorylation. As predicted from the known sensitivity of purified VH1 to drugs, OA did not inhibit the blockage of the IFN-γ signaling pathway, while sodium vanadate did. Restoration of p91-P levels by sodium vanadate was similar to that observed in the presence of the protein synthesis inhibitor CHX.

Strong evidence supporting a role for this viral phosphatase was obtained by infecting HeLa cells with a VV mutant in which expression of VH1 can be regulated. Levels of Stat1-P were inversely proportional to the amount of VH1 present in the infecting virions, indicating a clear correlation between the enzymatic activity and the suppression of an IFN-γ response. An alternative explanation might be that these virions are structurally defective. However, previous studies have shown that vindH1 virions produced by cells in the absence of IPTG show no aberrant characteristics other than the decreased level of VH1 (42). Analysis of purified virions by SDS-PAGE and silver staining revealed no alterations in protein composition, and at least one core-associated protein, F18, was present in equal amounts in wt, $H1^+$, and $H1^-$ virions (42). Entry of H1-deficient virions into cells is unaffected (42). Moreover, under conditions similar to those used here, synthesis of early transcripts is unaffected in cells infected with $H1^-$ virions, suggesting that the cores are not grossly defective (42). Thus, it seems unlikely that release of VH1 from the cores would be affected, although we cannot rule this out formally. The $H1^+$ virions, which contain substantially less VH1 than wt virus, are able to infect productively, showing that they do undergo uncoating. Nonetheless, their ability to block Stat1 activation is impaired. Given that dephosphorylation of Stat1 is greatly enhanced in VV-infected cells and that $NaVO_4$ is able to block the effect, the most likely explanation for our observations is that VH1 is responsible.

We were unable to detect a decrease in Stat1 phosphorylation by incubating purified VH1 with immunoprecipitated p91-P (data not shown), possibly owing to inaccessibility of the P-Tyr residues as a result of steric hindrance in preformed dimers. However, incubation of denatured p91-P with purified VH1 phosphatase resulted in dephosphorylation, indicating that, in vitro, Stat1 is a bona fide substrate for VV phosphatase. Several reports have identified viral substrates for the DS-PTPase VH1 (12, 41, 64), including the products of the A14 and A17 genes. Our present observations identify Stat1 as the first known cellular substrate for this viral enzyme.

Analysis of the catalytic mechanism employed by DS-PTPases showed that mutation of a critical Cys residue (21) could be used to produce catalytically inert DS-PTPs that retain their ability to bind to their substrates. We performed affinity chromatography in order to adsorb putative substrates to catalytically inactive mutants of VH1 coupled to a solid support. This approach permits the analysis of phosphoproteins that interact with the phosphatase (63). Using this method, we have identified Stat1 among the P-Tyr-containing proteins that specifically interacted with VH1, as determined by elution of $VH1^{C110S}$-bound proteins with the strong competitive inhibitor arsenate. These findings also indicate that $VH1^{C110S}$ can interact with various cellular phosphoproteins.

In summary, we describe an interference mechanism by

which VV inhibits the normal signaling pathway of IFN-γ to the nucleus. The results strongly suggest that VV phosphatase VH1 is the causative agent of this effect. These findings provide yet another example of the diverse mechanisms which VV, and viruses in general, have evolved to avoid immunosurveillance and establish persistence. Understanding the mechanisms by which viruses evade host defenses is essential for developing new strategies for the treatment of viral diseases and in the design of vaccines.

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