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IFN Regulatory Factor-1 Bypasses IFN-Mediated Antiviral Effects through Viperin Gene Induction

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Viperin is an antiviral protein whose expression is highly upregulated during viral infections via IFN-dependent and/or IFN-independent pathways. We examined the molecular alterations induced by the transcriptional activator IFN regulatory factor (IRF)-1 and found viperin to be among the group of IRF-1 regulated genes. From these data, it was not possible to distinguish genes that are primary targets of IRF-1 and those that are targets of IRF-1-induced proteins, like IFN-β. In this study, we show that IRF-1 directly binds to the murine viperin promoter to the two proximal IRF elements and thereby induces viperin expression. Infection studies with embryonal fibroblasts from different gene knock-out mice demonstrate that IRF-1 is essential, whereas the type I IFN system is dispensable for vesicular stomatitis virus induced viperin gene transcription. Further, IRF-1, but not IFN type I, mediates the induction of viperin transcription after IFN-γ treatment. In contrast, IRF-1 is not required for IFN-independent viperin induction by Newcastle disease virus infection and by infection with a vesicular stomatitis virus mutant that is unable to block IFN expression and secretion. We conclude that the IRF-1 mediated type I IFN independent mechanism of enhanced viperin expression provides a redundant mechanism to protect cells from viral infections. This mechanism becomes important when viruses evade innate immunity by antagonizing the induction and function of the IFN system. The Journal of Immunology, 2010, 184: 000–000.

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The online version of this article contains supplemental material.

Abbreviations used in this paper: CHX, cycloheximide; HCMV, human cytomegalovirus; HCV, hepatitis-C virus; IRF, IFN regulatory factor; IRF-E, IRF element; ISG, IFN stimulated gene; ISGF3, ISG factor 3; ISRE, IFN stimulated regulatory element; MEF, mouse embryonic fibroblasts; MOL, multiplicity of infection; NDV, Newcastle disease virus; VSV, vesicular stomatitis virus; wt, wild-type.

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that the activation of viperin expression can result through IFN-independent mechanisms, depending on the infecting virus (8, 9, 12). In this study, we describe the transcription factor IRF-1 as an essential mediator of viperin induction, acting through an IFN-independent pathway in vesicular stomatitis virus (VSV) infected cells. We also investigated the molecular basis for the IRF-1 induced promoter activation. Furthermore, we show that an enhanced viperin expression, comparable to that in VSV infected cells, results in a reduction of VSV replication even in the absence of functional IFN signaling.

Materials and Methods

Cell culture and treatments

IFNAR−/−, IRF-1−/−, and STAT1−/− mouse embryonic fibroblasts (MEFs) were kindly provided by R. Zawatzky (Deutsches Krebsforschungszentrum, Heidelberg, Germany), T. Decker (University of Vienna, Vienna, Austria), and M. Müller (Veterinary University of Vienna, Vienna, Austria). MEFs, NIH3T3, and myc/rafNIH3T3H1 cells (19) were grown in DMEM supplemented with 10% FCS and antibiotics. IRF-1/ER fusion protein was activated by 1 μM β-estradiol (Serva, Heidelberg, Germany). Cells were stimulated with 500 U/ml IFN-γ or 100 U/ml IFN-γ (PeproTech, Rocky Hill, NJ) for the indicated time periods. Treatment with cycloheximide (CHX) (60 μg/ml) occurred 2 h before IRF-1/ER activation and IFN-γ stimulation.

Viral infection

Cells were infected with Newcastle disease virus (NDV), VSV (Indiana strain), or AV2 for 1 h in DMEM without FCS and harvested at the indicated time points postinfection. AV2 is a VSV strain containing a mutated M-protein. For virus challenge, NIH3T3 cells were transiently transfected with pBUC5/6GON or a control plasmid using Metafectene (Biontex, Martinsried, Germany). Cells were infected 24 h after transfection, and the supernatant was collected 24 h postinfection. LMTK− cells were infected with serial dilutions of the supernatants to determine viral titers (PFU/ml) by a plaque-forming assay.

Vector constructs

The −1814/+14 viperin-promoter reporter construct was created by amplifying 1800 bp of the murine viperin promoter from IB10 cells (Primer: 5′-AAT TCC GTT CGA GCG GTA AOG GAC AGA ACA ACT GCA-3′ and 5′-AAT TCC CAAGT GTG GCG GTA ACA CAC CAC CTG C-3′). Xhol/HindIII digestion, and cloning in pGL3basic (Promega, Mannheim, Germany). Deletions were introduced into the −1814/+14 viperin-promoter construct by using the Quick Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according the manufacturer protocol. The sequences of primers are shown in Supplemental Table I. For construction of the viperin expression plasmid pBVC-3/6G/N, a control plasmid using Metafectene (Biontex, Martinsried, Germany), Cells were infected 24 h after transfection, and the supernatant was collected 24 h postinfection. LMTK− cells were infected with serial dilutions of the supernatants to determine viral titers (PFU/ml) by a plaque-forming assay.

Quantitative real-time PCR

Total RNA was extracted by using the RNeasy kit (Qiagen, Hilden, Germany) and reversely transcribed with Ready-To-Go You-Prime First-Strand Beads (Amersham Biosciences, Buckinghamshire, England). Quantitative RT-PCR analysis was performed by using a LightCycler (Roche, Mannheim, Germany) and the Quantitact SYBR Green PCR Kit (Qiagen). The data are represented as ratios relative to the results of α-actin. Standard curve analysis was performed for relative quantification. The following oligonucleotide primers were used: viperin, 5′-CTT CCA GAT GGA CCA GCA AGA C-3′ and 5′-CTT CCA GAT GGA CCA GCA AGA C-3′; IRF-1, 5′-CTC ACC AGG AAC CAC AGG AGG A-3′ and 5′-CTG AGT GGC TGT GAG CTC ACC A-3′; β-actin, 5′-TGG AAT CCT GTG GCG GTA ACC ACC ATC A-3′ and 5′-TGG AGT GGC TGT GAG CTC ACC A-3′; EcoRI/Sall digested, fused to the C terminus of a myc-tag, and cloned into pBVC-3 in front of an EMCV-derived IRES element, eGFP/neom fusion protein cassette.

 Luciferase Kit (Promega, Madison, WI). Signals were normalized to the Renilla luciferase activity. All experiments were performed at least twice with triplicates before calculating mean values and standard deviations.

Electro mobility shift assay

Murine IRF-1 and firefly luciferase were in vitro translated using the TNT T7 Quick Coupled Transcription/Translation System (Promega), according to the manufacturer’s protocol. pMT7-IRF-1 and luciferase T7 control DNA served as templates. EMSA experiments were performed with 5 μl in vitro translated protein mix in the absence or presence of 1 μg anti-IRF-1 (M-20; Santa Cruz Biotechnology, Santa Cruz, CA), as described in Klar and Bode (22). The used oligonucleotides were shown in the Supplemental Table II.

IFN assay

Type I IFN concentration in the cell culture supernatants was determined by an antiviral assay using LMTK− cells as described previously (23). Briefly, we seeded LMTK− cells in 96 wells and treated them for 24 h with serial diluted supernatants. Cells were challenged with VSF, IFN in the supernatant protects cells from the cytopathic effect through viral infection. Effects from supernatants were compared with standard type I IFN effect (PBL).

Promotor analysis

To predict potential transcription factor binding sites in the murine viperin promoter, the sequence from −1814 to +100 was analyzed by the web tool TESS (www.cbil.upenn.edu/tess). This program uses sites or consensus strings and positional weight matrices from the TRANSFAC, IMDB, and the CBIL-GibbsMat database.

Western blot analysis and Abs

Western blot analysis was accomplished according to standard procedures using ECL detection (Amersham Munich, Germany). The following primary Abs were used: IRF-3P (Ser396) (4D4G), Cell Signaling (Danvers, MA); VSV-G (PSD4), Boehringer Mannheim (Mannheim, Germany); and anti-Ab, Oncogene (Cambridge, United Kingdom). HRP-conjugated anti-rabbit and anti-mouse Abs (Amersham) were used as secondary Abs.

Results

Viperin transcription is upregulated by IRF-1

We previously established gene expression profiles to uncover the molecular alterations initiated by IRF-1 (24). Among several other genes with antiviral function, we found the viperin gene to be highly expressed in the presence of functional active IRF-1. This could be confirmed by transfection of a luciferase-based viperin promoter reporter in combination with an IRF-1 expression vector (Fig. 1A). Moreover, IRF-1 M6, a mutant that lacks the DNA binding domain, was incapable of inducing the promoter activity of the reporter construct (Fig. 1A), indicating that IRF-1 directly binds to the sequence and thereby activating the viperin promoter. To further verify this direct involvement of IRF-1 in the regulation of viperin, we analyzed viperin mRNA level in cells stably expressing an estradiol-activatable IRF-1hER fusion protein (19). Activation of the IRF-1hER fusion protein in the absence and presence of the translation inhibitor CHX strongly induced viperin mRNA synthesis (Fig. 1B, 1C). However, the presence of CHX prevented IFN-γ mediated upregulation of viperin supporting the hypothesis that under this condition IRF-1 gene expression must be induced to stimulate viperin transcription (Fig. 1C). Thus, activation of viperin transcription by sufficiently expressed IRF-1 is mediated directly and does not require de novo synthesis of proteins, enhancement of viperin expression can be mediated by elevated IRF-1 levels.

Bioinformatic promoter analyses of the murine viperin gene revealed several consensus response elements for NFκB, AP-1, NFAT, SP1, and three putative IRF elements (IRF-Es) (Fig. 2A). To determine the binding capacity of IRF-1 to these IRF-E sequences EMSAs were performed with in vitro translated IRF-1. As expected, the control oligonucleotide (IFN stimulated regulatory element [ISRE]) was efficiently bound by the IRF-1 protein
FIGURE 1. Viperin is an IFN-1 regulated gene. A. NIH3T3 cells were transiently transfected with a plasmid containing the Firefly luciferase gene driven by the wt viperin promoter (~1814/+14) and an expression vector coding for IRF-1 or IRF-1 M6, a mutant that lacks the DNA binding domain. Transfection efficiency was normalized by cotransfection of a constitutive Renilla luciferase expression plasmid. B, NIH3T3 cells, stably expressing an IRF-1-ER fusion protein (NIH3T3H), were treated with 1 μM β-estradiol to activate IRF-1. Relative viperin mRNA concentration was measured by quantitative real-time PCR at the indicated times. C, NIH3T3H cells were pretreated with 60 μg/ml CHX, 2 h before β-estradiol–induced activation of the IRF-1-ER fusion protein (IRF-1 and CHX) or IFN-γ (100 U/ml) treatment (IFN-γ and CHX). All data represent mean values with SD from at least two independent experiments.

FIGURE 2. IFN-1 directly binds to two proximal IRF-Es of the viperin promoter and thereby increases the promoter activity. A. Schematic map of the three IRF-1 binding sites (IRF-E-1, -2, and -3) in the 2000 bp upstream region of the murine viperin promoter. Their position in the promoter is represented with respect to the transcriptional start. B, Formation of IRF-1- DNA complexes was tested by EMSA using the sequence of the indicated IRF-E sites and in vitro translated IRF-1. A probe with the ISRE sequence of the IFN-β promoter was used as positive control. C, EMSA was performed as in B but with oligonucleotides representing mutated IRF-E sequences. D, NIH3T3H cells transiently transfected with a Renilla luciferase reporter plasmid (pBCRluc), a plasmid with the Firefly luciferase gene driven by the wt viperin promoter (~1814/+14) or reporter constructs containing mutations in one of the IRF-E sites. Activation of IRF-1 by β-estradiol (1 μM) treatment was performed 24 h before harvesting cells for the dual luciferase assay. Relative luciferase activities of Firefly luciferase were calculated by normalization to Renilla luciferase activity. The graph shows the fold increase of relative luciferase activities in cells treated with β-estradiol (IRF-1) compared with untreated cells (C).

IRF-1 is essential for IFN-γ and VSV induced viperin expression

Viperin is highly induced on IFN stimulation and by infection with a broad range of viruses. To investigate whether IRF-1 exerts influence on these events, we tested wild-type (wt) and IRF-1-deficient (IRF-1-/-) MEFs for alterations in viperin mRNA levels caused by IFN-β, IFN-γ, VSV, and NDV, respectively. As expected, in wt MEFs, increased viperin mRNA levels could be detected as a consequence of treatment with all four stimuli (Fig. 3A). Stimulation of IRF-1-/- cells with IFN-β or infection with NDV leads to the induction of viperin (Fig. 3B) indicating that IRF-1 plays no role under these conditions. In contrast, IFN-γ failed to induce viperin expression in IRF-1-/- cells, suggesting an important role of IRF-1 in IFN-γ mediated viperin induction. A more revealing finding was that in the absence of the IRF-1 gene activity, the VSV-mediated viperin induction failed (Fig. 3B), indicating that the presence of the IRF-1 is essential to activate the viperin promoter under these conditions.

IFN-γ is known to be a potent IRF-1 inducer (25). Its binding to the IFN-γ receptor activates STAT1 and NFκB, which synergistically interacts to induce IRF-1 transcription (26). Therefore, we examined whether STAT1 also plays a role in the IRF-1-mediated viperin induction on virus infection. First of all, we investigated whether STAT1 itself or synergistically with IRF-1 influences the viperin promoter activity. Transfection of NIH3T3 cells with an IRF-1 expression vector induced the viperin promoter activity in a dose-dependent manner, whereas expression of STAT1 had no significant influence, both in the absence or presence of ectopic IRF-1 (Fig. 4A). Therefore, a direct influence of STAT1 on the viperin promoter activity is unlikely. In addition, performed quantitative RT-PCR analyses of STAT1-/- MEFs showed only weak induction of viperin by NDV but no induction after VSV infection (Fig. 4B). In contrast, the transient expression of IRF-1 significantly enhanced the amount of viperin mRNA in STAT1-deficient MEFs (Fig. 4C). This supports our hypothesis that an increase of cellular IRF-1 expression is sufficient to activate the viperin promoter and that STAT1 is essential for stimulation of IRF-1 transcription. These results also indicate a STAT1-dependent mechanism of viperin induction during NDV infection, that results from the involvement of STAT1 in other IRF-1 independent antiviral signaling pathways, namely, the type I IFN pathway.
Infect IFNAR

Increased IRF-1 mRNA concentrations were detected in VSV in-

A

infection independent of type I IFN (Fig. 5).

Type I IFN signaling (Fig. 5).

An intact IFN system is dispensable for the enhanced expression and the antiviral function of viperin in VSV infected cells

Boudinot et al. showed that viperin expression in a dendritic cell line infected with VSV is upregulated even in the presence of Abs neutralizing type I IFN activity, namely, in an IFN-α/β independent manner (9). The results presented in Fig. 3 reveal that IRF-1 is essential for IFN-γ and VSV induced viperin expression. To assess the possible involvement of IRF-1 in an IFN independent regulation of viperin, type I receptor deficient (IFNAR−/−) MEFs were cultured in the presence of IFN-β, IFN-γ, or infected with VSV and NDV, and analyzed for changes in viperin mRNA concentration. We can ex-

clude an IFN effect dependent on IFN-γ, because only T, NKT, and NK cells are able to express IFN-γ. The IRF-1–dependent viperin induction by IFN-γ and VSV was still detectable in the absence of type I IFN signaling (Fig. 5A). Interestingly, NDV also activated viperin expression independent of type I IFN (Fig. 5A). In addition, increased IRF-1 mRNA concentrations were detected in VSV infected IFNAR−/− MEFs, but mRNA levels were only marginally but statistically significant changed by NDV infection (Fig. 5B).

These findings suggest that infected cells can respond to both viruses via IFN independent mechanisms. However, the IRF-1 dependent pathway is indispensable only in cells infected with VSV.

To demonstrate the relevance of the VSV-induced IRF-1 de-

pendent pathway, we examined whether the induction of viperin is adequate to counteract viral replication. The wt and IFNAR−/− MEFs, transiently transfected with an expression vector encoding for viperin or a control vector, were infected with VSV for 1 h. In the 24-h postinfection we analyzed viral replication by quantifying the concentration of infectious viruses in the supernatant. The expression of viperin led to a strong antiviral response against VSV, in both wt- and IFNAR-deficient cells (Fig. 5C, Supplemental Fig. 1).

The inhibition of viral replication was comparable to the effect of IFN treatment. Depletion of type I IFN in the supernatant by specific Abs prevented the IFN induced but not the viperin induced inhibition of viral replication. Generally, VSV titer in IFNAR−/− were higher compared with those in wt cells, confirming the role of IFN in antiviral defense. Nevertheless, this experiment reveals viperin to be very effective in diminishing VSV replication, even in the absence of functional IFN signaling.

IRF-1 provides a bypass mechanism of viperin induction during infection with viruses that counteract the IFN system

As many other viruses, VSV evolved mechanisms to circumvent the innate immune defense by blocking the IFN signaling (27). Whereas activation of IRF-3 and induction of IFN-β expression were found in VSV and AV2 infected cells, IRF-7 mRNA expression was not detectable in VSV infected cells (Supplemental Fig. 2). It seems that the IRF-1 dependent pathway of viperin induction is of particular importance when cells cannot activate the IFNAR response on infection. Conversely, the IRF-1 mediated induction of viperin expression should be dispensable when viruses fail to block the IFN signaling. To test this hypothesis, IRF-1−/− MEFs were infected with a mutant VSV strain (AV2), which lacks the ability to suppress IFN gene expression (28). AV2 and NDV, but not wt VSV, infected cells responded with substantial secretion of IFN (Fig. 6A). Consistently, an increase of viperin mRNA, comparable to that of NDV infected MEFs (Fig. 3B), was measured on AV2 infection (Fig. 6B), showing that the capability of VSV to prevent IFN expression forces the cell to induce viperin via an IFN independent, but IRF-1 dependent pathway.

Discussion

A multiplicity of viruses, LPSs and dsRNA induce the viperin expression through IFN-dependent and IFN-independent mechanisms. The IFN-dependent viperin induction can be mediated by
The budding of the virus from the plasma membrane. This is (10). In contrast, in influenza A virus infected cells, viperin inhibits several structural proteins critical for viral assembly and maturation which viperin limits the viral replication is unknown. In HCMV pseudotyped HIV (14), and HCV (15). The exact mechanism by induced ISGF3 complex are very similar.

Tonic activation of IRF-1 by binding to conserved IRF elements in the promoter of its target genes. Our data demonstrate that IRF-1 exerts a direct antiviral response, which is consistent with preventing vaccinia virus replication (30). In addition, it was found that IRF-1 exerts a direct antiviral response, which is consistent with a consequence of viperin-induced disruption of lipid-raft microdomains, which results from the interaction and inactivation of the enzyme farnesyl diphosphate synthase with viperin (11). VSV does not bud from lipid rafts, which is indicative for the presence of other antiviral mechanisms. Our data show, that viperin expression also inhibits the replication of VSV more than 1000-fold (Fig. 5C). This strong inhibition of virus replication by an intrinsic effect is only comprehensible in the unlikely case of 100% transfection efficiency. Transfection efficiency was determined by the cotransfer of a GFP expression vector and is 60% or higher (data not shown). This transfection efficiency is not sufficient for a 1000-fold reduction of viral replication. One possible explanation is that a very low expression level of viperin that might be induced by a transfection of nearly 100% of cells is sufficient to induce an antiviral response. Alternatively, paracrine effects could be responsible for this phenomenon. We checked the supernatant for further antiviral effects but could not detect a respective activity (data not shown).

Previous studies with IRF-1 deficient MEFs demonstrated an involvement of IRF-1 in the type I IFN-dependent antiviral response against ECMV infection (29) and in an IFN-γ mediated mechanism preventing vaccinia virus replication (30). In addition, it was found that IRF-1 exerts a direct antiviral response, which is consistent with

type I IFNs, IFN-γ, and type III IFNs. Previous publications showed that activation of the type I IFN signaling pathway enhances the viperin transcription through the STAT1/STAT2/IRF-9 complex termed ISG factor 3 (ISGF3) by binding to the promoter response element ISRE (8, 12). In this article, we reveal IRF-1 as a mediator for the VSV and IFN-γ amplified viperin expression (Fig. 3). IRF-1 mediates its activity by binding to conserved IRF elements in the promoter of its target genes. Our data demonstrate that the viperin promoter contains two IRF-E sites, which are necessary for IRF-1 mediated promoter activation (Fig. 2). This overlap of gene induction by IRF-1 and type I IFNs is not astonishing, because the IRF binding sites and the one from IFN-induced ISGF3 complex are very similar.

Viperin limits replication of viruses like HCMV (10), VSV-pseudotyped HIV (14), and HCV (15). The exact mechanism by which viperin limits the viral replication is unknown. In HCMV infected cells the expression of viperin inhibits the expression of several structural proteins critical for viral assembly and maturation (10). In contrast, in influenza A virus infected cells, viperin inhibits the budding of the virus from the plasma membrane. This is
the finding that enforced expression of IRF-1 prevents VSV, EMCV, and HCV replication in an IFN-independent way (31, 32). Because our analysis revealed IRF-1 as being an essential factor for the increase of viperin gene expression postinfection with VSV, a virus that is extremely efficient in blocking the type I IFN function (Fig. 6A), we conclude that the IFN-independent induction of viperin is mediated by IRF-1 and propose a model that is depicted in Fig. 7. Virus infection (e.g., NDV) can lead to the production and secretion of IFN-β. In turn, IFN-β interacts with type I IFN receptors in an autocrine and paracrine manner, inducing ISGs via the JAK/STAT pathway. The independently induced IRF-1 induces the viperin expression as well (Fig. 1). Because both pathways are in place at the same time induction of viperin by NDV can neither be blocked by the deficiency of IFNAR nor the lack of IRF-1 (Figs. 3B, 5A). This model also explains the role of STAT1 that is essential for NDV and VSV infection because it is involved in both pathways (Fig. 4B). VSV infection blocks the production of IFN-β in wt- and IRF-1−/− cells (Fig. 6A and data not shown). Thus, viperin expression cannot be induced by the JAK/STAT pathway but is dependent on the alternative IRF-1 pathway (Fig. 3F). If the block of the IFNAR signaling in VSV can be overcome by using the VSV mutant strain AV2, which lacks the inhibitory M protein, the induction of viperin is no longer strictly dependent on IRF-1 but can use the IFNAR signaling pathway (Fig. 6B). Thus, IRF-1 provides a redundant mechanism to cope with VSV infections under those conditions leading to an IFN-mediated immune response. However, most viruses have evolved strategies to counteract the IFN system, therefore IFN-independent antiviral pathways are crucial for the defense against these infections. During viral infection, recognition of different viral structures leads to the activation of IRF-3 and the production of type 1 IFNs. Many viruses encode proteins that modulate one or both of these two pathways (18). Essentially, host cells have to exhibit response mechanisms that are independent of IRF-3 and/or IFN-signaling pathways to sense and combat viral infections. Some reports described an early immune response mechanism independent of type 1 IFNs, IRF-3, and all common pattern recognition receptors (33). The entry of UV inactivated VSV particles results in the expression of a subset of ISGs by an IFN-independent but IRF-3 dependent mechanism. Interestingly, only infections with a replication competent virus promote an efficient VSV-neutralizing immune response (34, 35), suggesting that additional mechanisms besides the IRF-3 pathway are involved in VSV defense. The failure of an elevated viperin expression in IRF-1−/− MEFs infected with VSV, clearly shows that one of those additional mechanisms can be mediated by IRF-1 (Fig. 3B). Further investigations have to clarify whether the virus mediated IRF-1 dependent activation of a single antiviral protein (viperin) is indicative of a general fail-safe pathway for the host against viral infection. It is tempting to speculate that viperin is only one of the antiviral proteins induced by IRF-1, suggesting that the IRF-1-mediated bypass described in this study applies for other viral infections as well.

In summary, our study demonstrates that the VSV-mediated viperin induction occurs independently of IFN, through IRF-1 and that the transcriptional upregulation of viperin is sufficient to reduce VSV replication. We identified two proximal IRF-3 sites that are essential for the IRF-1 mediated enhancement of the viperin promoter activity. In addition, our studies show that IRF-1 plays a pivotal role in host response and suggest that it defends the cells when viruses inhibit the IFN signaling.

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Disclosures

The authors have no financial conflicts of interest.

IFN INDEPENDENT, IRF-1 DEPENDENT VIPERIN INDUCTION

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