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ORIGINAL ARTICLE

Host factor Ebp1 inhibits rinderpest virus transcription in vivo

M. Gopinath · S. Raju · A. Honda · M. S. Shaila

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Abstract ErbB3 binding protein Ebp1 has been shown to downregulate ErbB3 receptor-mediated signaling to inhibit cell proliferation. Rinderpest virus belongs to the family *Paramyxoviridae* and is characterized by the presence of a non-segmented negative-sense RNA genome. In this work, we show that rinderpest virus infection of Vero cells leads to the down-regulation of the host factor Ebp1, at both the mRNA and protein levels. Ebp1 protein has been shown to co-localize with viral inclusion bodies in infected cells, and it is packaged into virions, presumably through its interaction with the N protein or the N-RNA itself. Overexpression of Ebp1 inhibits viral transcription and multiplication in infected cells, suggesting that a mutual antagonism operates between host factor Ebp1 and the virus.

Introduction

Rinderpest virus (RPV) belongs to the genus *Morbillivirus* in the family *Paramyxoviridae*, a family of non-segmented negative-sense RNA viruses that also includes certain human pathogens such as measles virus and parainfluenza virus. The viral genome is 15,882 bases in length, coding for structural proteins in the following order: 3′-leader-N–P-M-F–H–L-trailer-5′ [4]. The viral genomic RNA is encapsidated by the nucleocapsid protein N to form a helical N-RNA that serves as the template for both transcription and replication [9]. During transcription, the viral N-RNA directs the synthesis of all species of viral mRNA by the virion-associated RNA-dependent RNA polymerase (L-P complex), which are translated to produce viral proteins. During replication, a full-length complement of the genomic RNA is made and encapsidated by the newly synthesized N protein, which in turn acts as the template for the synthesis of viral genome.

Ebp1 was originally identified as an ErbB3-binding protein that gets translocated to the nucleus upon receptor activation by heregulin [24]. Knockdown of Ebp1 in mice as well in *Arabidopsis thaliana* results in a dwarfism phenotype at earlier stages of growth, suggesting the involvement of Ebp1in development [13, 26]. Ebp1 has been shown to be downregulated in many cancer cell types, and overexpression of Ebp1 in breast and prostate cancer cell lines leads to the inhibition of cell growth by promoting G2/M cell-cycle arrest. Ebp1 exerts its anti-proliferative activity by repressing the transcription of E2F1-regulated cell-cycle-associated genes in association with RB, HDAC2 and Sin3A, and Ebp1 phosphorylation at Ser 363 has been shown to be essential for this function [10]. In addition, Ebp1 was also shown to bind RNA by its association with the bcl-2 mRNP complex and FMDV IRES in conjunction with polypyrimidine-tract-binding protein (PTB) [6, 19]. However, a role for Ebp1 in regulating virus replication was reported recently by Honda et al. [12], who demonstrated the selective inhibition of influenza virus transcription by Ebp1, both in infected cells and in vitro, without affecting the cap-binding and endonuclease properties of the viral polymerase. In addition, overexpression of

M. Gopinath · S. Raju · M. S. Shaila (✉)
Department of Microbiology and Cell Biology,
Indian Institute of Science, Bangalore 560012, India
e-mail: shaila@mcbl.iisc.ernet.in

A. Honda
Faculty of Engineering, Frontier Biosciences,
Hosei University, Koganei, Tokyo 184-8584, Japan

Present Address:
M. Gopinath
Institute of Medical Biology, 8A, Biomedical Grove,
Biomedical Science Institute, Singapore 138648, Singapore

Ebp1 was shown to result in reduction of virus titers. Ebp1 was shown to be localized to the nuclear membrane in association with the PB1 subunit of the polymerase in infected cells [11, 12]. However, there are no reports on the role of Ebp1 on the transcription process in members of the order *Mononegavirales*. In the present work, we show that Ebp1 is downregulated upon RPV infection, and overexpression of Ebp1 inhibits genome transcription as well as virus replication, suggesting an antagonistic role of Ebp1 in the negative-sense RNA virus life cycle.

Materials and methods

Cells, viruses and antibodies

The Vero cell line, an African green monkey kidney cell line, was obtained from the National Centre for Cell Science, Pune, India, and maintained in DMEM supplemented with 10% fetal calf serum. *Spodoptera frugiperda* (Sf21) insect cells were obtained from the National Centre for Cell Science, Pune, India, and maintained in TC-100 medium (GIBCO-BRL, USA) with 10% fetal calf serum. A tissue culture-adapted vaccine strain, RBOK, which is the original attenuated Kabete 'O' strain of RPV, was obtained from the Institute for Animal Health and Veterinary Biologicals, Bangalore, India. Polyclonal antibodies were prepared by immunizing rabbits with recombinant Ebp1 protein purified from extracts of Sf21 cells infected with recombinant baculovirus expressing Ebp1 [12]. A mouse monoclonal antibody specific for the nucleocapsid protein of RPV (F8D3) generated earlier in the laboratory (Suresh K.B., Rajasekhar M. and Shaila, M.S., unpublished) was employed in an immuno-colocalisation experiment. For virus production, confluent Vero cells were infected with RPV at an moi of 1, and virus released into the supernatant and cell-associated virus were pooled after 96 h of incubation at 37°C and purified according to Ghosh et al. [8].

Ebp1 mRNA levels in RPV-infected cells

Vero cells in 35-mm dishes were infected with RPV at an MOI of 5. At 0, 12, 24, 36, 48 and 60 h postinfection, total RNA that was free from genomic DNA was isolated using SV Total RNA Isolation System (Promega, USA) according to the manufacturer's instructions. Equal amounts of total RNA (4 μg) were resolved by 1.5% MOPS-formaldehyde agarose gel electrophoresis, transferred to a nylon membrane and UV cross-linked. Generation of ^{32}P-labeled negative-sense RNA probes against RPV N and H mRNA has been described earlier [9]. For making a cDNA probe against Ebp1, Ebp1 ORF was released from pCAGGS-Ebp1 by EcoRI digestion and labeled with [α-^{32}P] ATP using a Hexalabel^{TM} DNA Labeling Kit (Fermentas, USA). Unincorporated label was removed from the probe by passing it through Sephadex G50 columns. The Ebp1 cDNA probe was then denatured at 95°C for 5 min and hybridized to the blot at 60°C overnight in hybridization buffer (50% formamide, 6X SSC, 1% SDS, 5X Denhardt's solution, 50 μg/ml of sheared herring sperm DNA). The hybridized blot was exposed to phosphorimaging plates after washing three times with 0.5X SSC and 0.1% SDS.

For real-time quantification of Ebp1 mRNA, 2 μg of total RNA was reverse transcribed in 1X RT buffer (Fermentas) containing 2 mM dNTP and 20 U of human placental RNAse inhibitor. The cDNA was further diluted tenfold with water, and 2 μl of the diluted cDNA was used along with the Ebp1-specific primer pair (Ebp1F: 5′-GA CGAGCAACAGGAGCAAACT-3′ and Ebp1 R- 5′-CT TCCACCAAGGACCGAAGT-3′) and SYBR Green qPCR master mix in a total reaction volume of 10 μl. To normalize gene expression, a parallel amplification of an endogenous (GAPDH) gene was performed with specific primers (GAPDHF: 5′-CATGAG AAGTATGACAA CAGCCT-3′ and GAPDHR: 5′-AGTCCTTCCACGAT ACCAAAGT-3′). Real-time PCR was carried out in an ABI Prism 7700 (PE Applied Biosystems). Relative quantification of Ebp1 was performed by comparing the ΔCt values after normalizing against GAPDH control, using the SDS 2.1 software (ABI Prism). The results are the average of three independent experiments.

RPV and Ebp1 protein levels in infected cells

Vero cells were infected with RPV as above, and cells were lysed in RIPA buffer containing 50 mM Tris HCl, pH 7.4, 300 mM NaCl, 1% Triton X 100, 1% sodium deoxycholate, 10% glycerol and 1X protease inhibitor cocktail at the indicated time points. Fifty μg of the clarified lysate was resolved by 10% SDS–PAGE, transferred to a nitrocellulose membrane (GE Healthcare, USA) and probed either with rabbit anti-Ebp1 (1:1,000) or rabbit hyperimmune sera against whole virus (1:1,000) or mouse anti-actin (1:1,500). Blots were washed with PBST (PBS with 0.25% Tween 20) and incubated with anti-rabbit/mouse antibody conjugated to HRP (1:5,000). After washing three times with an excess volume of PBST, proteins were detected by developing the blot with enhanced chemiluminescence reagent (GE Healthcare, USA).

Indirect immunofluorescence and confocal microscopy

Vero cells were grown on tissue culture chamber slides (Nunc) to 50% confluence and infected with RPV at an moi of 5. Forty-eight hours after infection, cells were washed

once with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature (RT). Cells were permeabilized in PBS containing 0.2% Triton X-100 for 5 min at RT. The cells were incubated in blocking buffer (5% BSA in PBS) for 1 h at RT and washed three times with PBS. Primary antibodies were added to cells in PBS containing 3% BSA. The primary antibody combinations were rabbit anti-Ebp1 (1:100) and mouse hyperimmune sera against whole virus (1:50) or mouse monoclonal antibody for RPV N (1:100). Cells were washed three times with PBS and incubated with TRITC-labeled anti-mouse antibody and FITC-labeled anti-rabbit antibody. Cells were visualized using a Zeiss Laser scanning microscope LSM 510.

Co-immunoprecipitation

RPV-infected total cell extracts at 12, 24 and 48 h post-infection (hpi) were pretreated with 20 µl of 10% protein A Sepharose CL-4B beads (GE Healthcare, USA) for 1 h at 4°C. Co-immunoprecipitation was carried out with anti-Ebp1 antibodies (1 µl) in the presence of 30 µl of protein A Sepharose at 4°C for 4 h. As a control, an extract prepared at 48 hpi was subjected to co-immunoprecipitation with pre-immune serum. The immunoprecipitated complex was washed three times with buffer containing 50 mM Tris–HCl, pH 7.4, 300 mM NaCl, 0.1% NP40, 0.05% sodium deoxycholate and 1 mM PMSF. Beads were suspended in 1X SDS–PAGE loading buffer, boiled for 5 min and loaded onto a 10% SDS–PAGE gel. Western immunoblotting was carried out with rabbit hyperimmune sera against RPV.

Effect of Ebp1 overexpression on viral mRNA/protein synthesis in infected cells

Vero cells were transfected with increasing concentrations (0.5, 1 and 2 µg) of the pCAGGS-Ebp1 construct or with pCAGGS vector alone (1 µg) using Cellfectin transfection reagent (Gibco BRL, USA) as per the manufacturer's protocol. At 24 h posttransfection, cells were either mock infected or infected with RPV at an MOI of 5. At 36 h postinfection, cells were washed once with PBS, and total cell extract or total cellular RNA was prepared as described earlier.

Effect of Ebp1 overexpression on virus production

Vero cells were transfected with pCAGGS-Ebp1 or pCAGGS vector alone as mentioned above, and at 24 h posttransfection, cells were infected with RPV at an MOI of 1. Ninety-six hours after infection, infected cells and supernatants were subjected to three rounds of freeze–thaw cycles to release the cell-associated virus. Cellular debris was clarified by centrifugation at 10,000*g* for 10 min at 4°C. Supernatant containing the virus was titred by the serial endpoint dilution method, and $TCID_{50}$ values were calculated by employing the Reed and Muench formula [7].

Results

Ebp1 levels are downregulated upon RPV infection

To test the effect of rinderpest virus infection on Ebp1 expression, Vero cells were infected with RPV. At different time points, equal amounts of total RNA were loaded onto a formaldehyde agarose gel (Fig. 1a) and subjected to northern blot analysis using Ebp1-specific cDNA probes. The northern blot revealed the presence of a single transcript of 1.6 kb in size, corresponding to the full-length Ebp1 mRNA (Fig. 1b). Upon RPV infection, a gradual downregulation of Ebp1 transcription was observed, which was maximal at 60 hpi. Corroborating results were also obtained when Ebp1 mRNA was quantified by Q-RT PCR (Fig. 1c). Further, total cell extracts from infected cells at different time points were electrophoresed on an SDS–polyacrylamide gel and immunoblotted with antibodies specific for Ebp1. In agreement with the northern blot results, the amount of Ebp1 protein was reduced upon virus infection as early as 24 hpi, which corresponds to the onset of viral protein synthesis (Fig. 1d, e). At 60 hpi, Ebp1 levels were less than 40% of that of the initial time point (0 hpi). Under these conditions, the level of a cellular protein, actin, did not change (Fig. 1f).

Ebp1 protein co-localizes with the RNP complex

Ebp1 has been shown to interact with many cellular proteins, such as Akt, Pak1, Mybbp1a, HDAC2 and Sin 3A [3, 23, 27]. In addition, the interaction between Ebp1 and polypyrimidine tract-binding protein (PTB) was shown to be crucial for FMDV IRES activity [19]. Honda et al. [12] demonstrated that Ebp1 interacts with the PB1 subunit of influenza virus RNA polymerase and that the PB1 interaction site on Ebp1 overlaps with the binding sites for cellular Akt. Hence, the ability of Ebp1 to interact with rinderpest virus proteins was tested by immuno co-localization assay. Virus-infected cells were probed with rabbit anti-Ebp1 antibody and mouse hyperimmune sera against the virus at 48 hpi. Ebp1 was seen to co-localize with viral inclusion bodies, where active transcription and replication occur (Fig. 2b). In addition, co-localization was also observed when anti-N antibody was used instead of hyperimmune sera (Fig. 2c), further confirming that there is a specific interaction between Ebp1 and the RNP complex in the cytoplasm. No fluorescence signal was observed

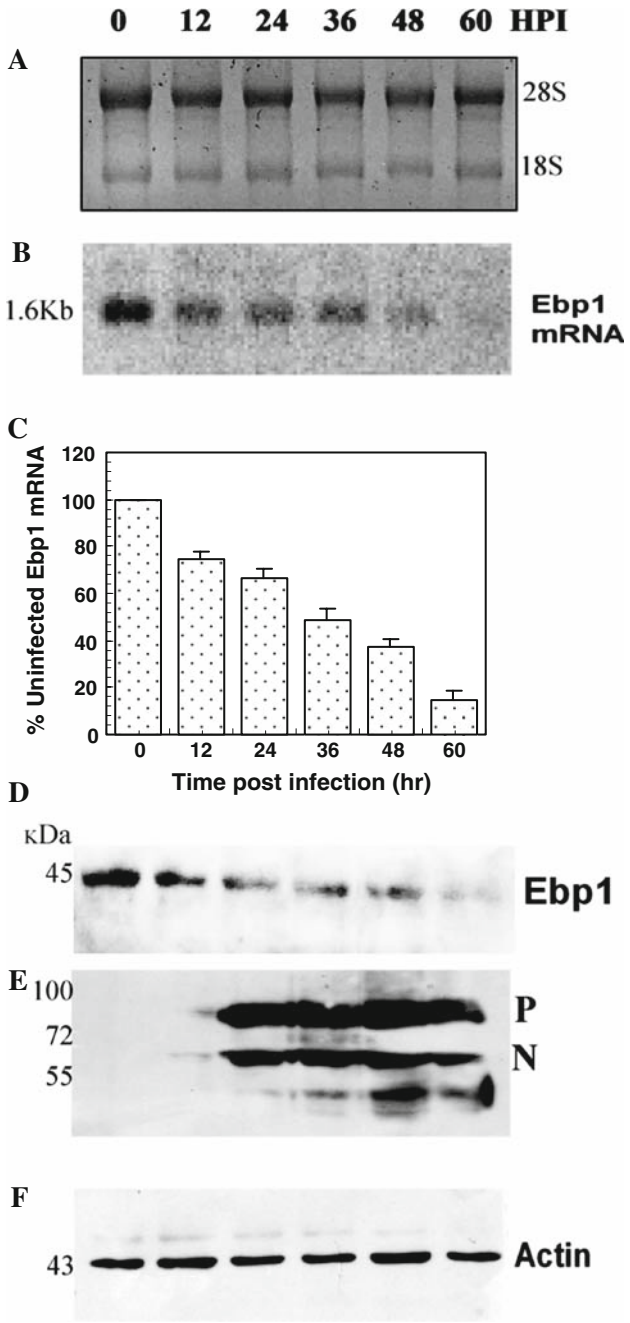

Fig. 1 Ebp1 mRNA and protein levels in RPV-infected cells. Vero cells were infected with RPV, and at indicated number of hours postinfection (hpi), total RNA or total cell extracts were prepared as described in "Materials and methods". Total RNA was subjected to formaldehyde-agarose gel electrophoresis and stained with ethidium bromide to verify equal loading of total RNA (**a**). Northern blot analysis of Ebp1 mRNA (**b**). Real-time PCR analysis of Ebp1 mRNA levels in infected cells at the indicated time points. The data are an average of three independent experiments normalized to endogenous GAPDH levels (**c**) Total cell extracts prepared from RPV-infected cells were resolved through 10% SDS–PAGE and western immunoblotted with anti-Ebp1 (**d**), hyperimmune serum against the whole virus (**e**) or anti-actin monoclonal antibodies (**f**) and detected by enhanced chemiluminescence

when uninfected cells were probed with viral-protein-specific antibodies (Fig. 2a). Co-localization of Ebp1 with viral inclusion bodies suggested that it could be packaged into mature virions. Hence, virions were purified from culture supernatants of RPV-infected cells by ultracentrifugation as described earlier [8]. The 100,000*g* supernatant and the virus pellet were subjected to polyacrylaminde gel electrophoresis and western blotting, using antibodies to Ebp1 and viral protein N. As expected, Ebp1 was found to be present in purified virion, indicating that it is packaged into mature virus, possibly through interaction with viral protein/s (Fig. 2d). The S100 fraction showed the presence of N protein (presumably monomeric), which was released during cell lysis.

However, immuno-colocalization could not be used to clearly distinguish which one of the viral proteins interacts with Ebp1 due to strong intermolecular interactions among the viral N, P and L proteins in infected cells. Hence, a co-immunoprecipitation assay was performed with RPV-infected cell lysate at 48 h postinfection using anti-Ebp1 antibodies. The immunoprecipitated complex was subjected to western blot analysis with a hyperimmune serum against the whole virus. Ebp1 was found to interact with RPV N protein in virus-infected cells as early as 24 h postinfection. This interaction was specific, since the 48 hpi lysate immunoprecipitated with pre-immune sera did not pull down the N protein (Fig. 2e).

Overexpression of Ebp1 inhibits RPV transcription and reduces virus yield in vivo

The results described above indicate that RPV infection downregulates the expression of a key cellular protein, Ebp1, which associates with the transcription-replication machinery of the virus. The results also point to the likelihood of Ebp1 having an effect on the multiplication of virus in infected cells. Therefore, the effect of Ebp1 overexpression on genome transcription and virus replication was tested in vivo. Vero cells were transfected with increasing concentrations of pCAGGS-Ebp1 construct in which Ebp1 is cloned under a constitutive CMV promoter. Increasing the concentration of pCAGGS-Ebp1 led to increased synthesis of Ebp1 mRNA (Fig. 3a). Endogenous Ebp1 mRNA could not be detected in cells transfected with the vector (pCAGGS) alone, possibly due to low level of expression, which may not get detected when the exposure time is shorter. Overexpression of Ebp1 at the time of viral infection (Fig. 3a) led to more than a twofold reduction in N mRNA synthesis in infected cells (Fig. 3b). A stronger effect on H mRNA synthesis was observed (Fig. 3c), suggesting that Ebp1 inhibits overall transcription of viral mRNA, and this inhibition is not gene-specific. Such

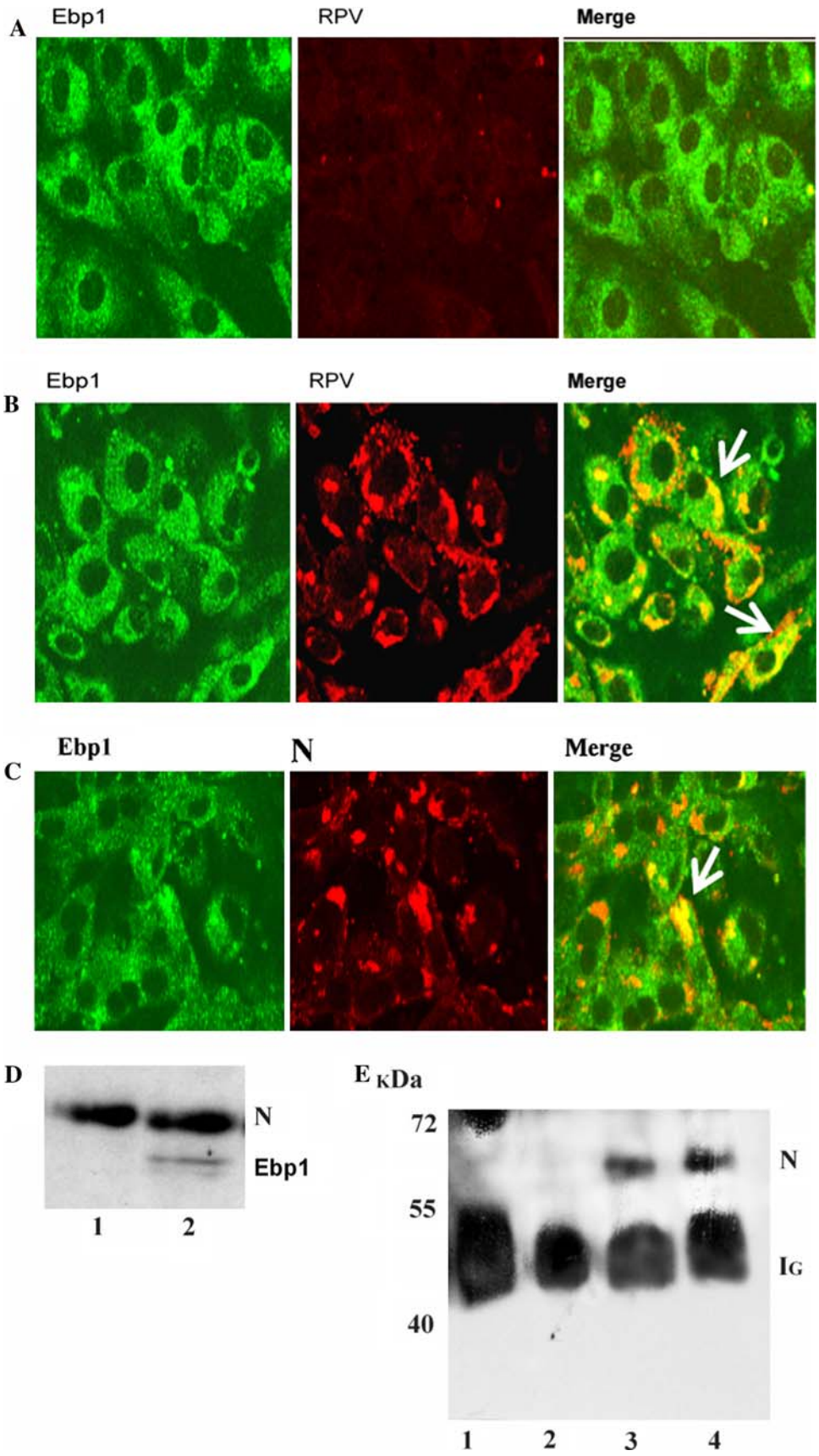

Fig. 2 Ebp1 interaction with RPV N/N-RNA. Vero cells were either mock infected (**a**) or infected with RPV (**b**, **c**), and at 48 h postinfection, cells were subjected to immunocytochemical analysis with rabbit anti-Ebp1 and mouse polyclonal sera against purified virus (**a**, **b**) or rabbit anti-Ebp1 and mouse monoclonal antibody against RPV N protein (**c**). Rinderpest virus was purified from infected culture supernatant by ultracentrifugation. The virus pellet (lane 2) and S100 supernatant fraction (lane 1) were probed with a mixture of anti-Ebp1 and anti-N antibodies (**d**). **e** Clarified lysate from Vero cells infected with RPV at 12, 24, and 48 hpi (lanes 2–4) was immunoprecipitated with anti-Ebp1 antibodies, and the immunoprecipitate was electrophoresed and western immunoblotted with hyperimmune sera against whole virus. As a control, Co-IP was performed using pre-immune sera with lysate collected at 48 hpi (lane 1). IgG, antibody heavy chain. *White arrows* indicate the areas of co-localization

inhibition was not seen in cells transfected with vector alone.

The overall transcriptional downregulation of RPV viral mRNA synthesis may result in the reduction of viral protein synthesis, which in turn may lead to decreased virus production. In order to test this hypothesis, viral proteins synthesis was tested in Ebp1-overexpressing cells by western blotting. Transfection of increasing amounts of pCAGGS-Ebp1 led to increased expression of Ebp1 (Fig. 4a). As expected, overall down regulation of viral protein synthesis (N, P, M and H) was observed in Ebp1-transfected cells compared to those transfected with the vector alone (Fig. 4b). L could not be detected in total cell extract due to its low expression levels. In addition, overexpression of Ebp1 resulted in a two-log reduction in RPV titers, suggesting that Ebp1 may play an antagonistic role against RPV infection in infected cells (Fig. 4d).

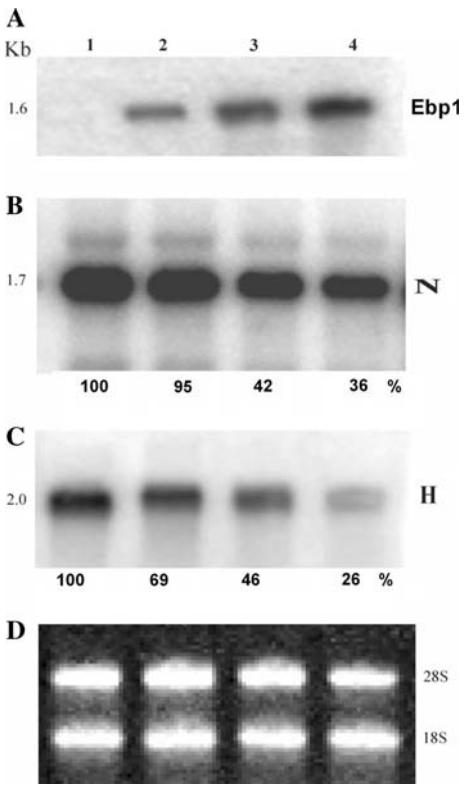

Fig. 3 Ebp1 overexpression inhibits virus transcription. Vero cells were transfected with increasing concentrations (0.5, 1 and 2 μg) of pCAGGS-Ebp1 (lanes 2–4) or 1 μg of pCAGGS vector alone (lane 1). At 24 h posttransfection, cells were infected with RPV at an MOI of 5. At 36 h postinfection, total RNA was probed for Ebp1 (**a**), RPVN (**b**) or RPV H(**c**) mRNA by northern blotting, using gene-specific probes. **d** Total RNA was stained with ethidium bromide to ensure equal loading

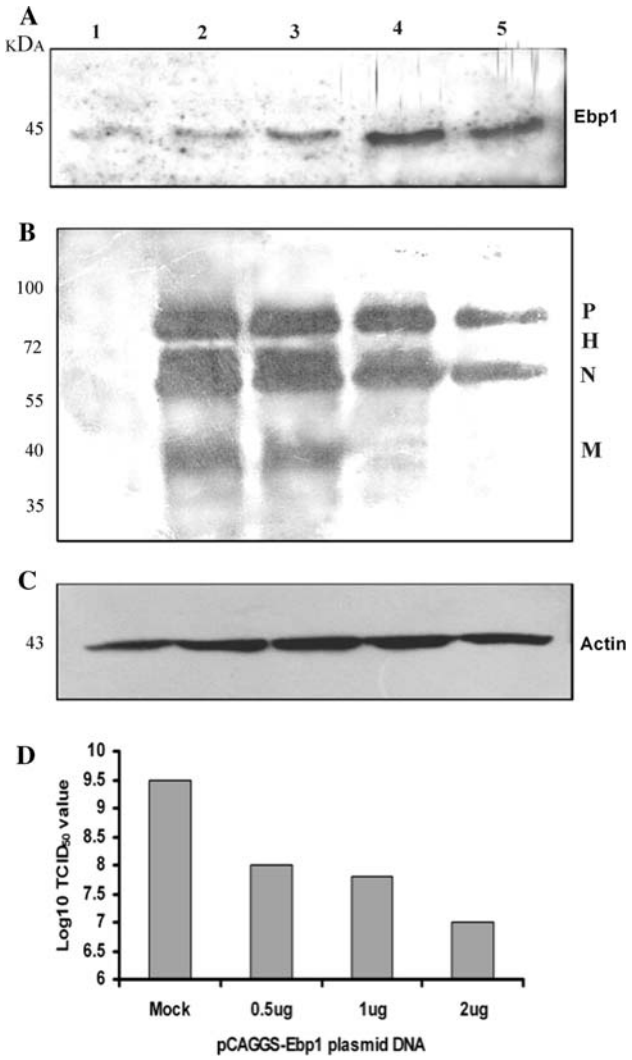

Fig. 4 Ebp1 overexpression inhibits virus protein synthesis and multiplication in vivo. Vero cells were transfected and infected with RPV as shown in Fig. 3. *Lane 1* represents cells that are neither transfected nor infected. Total cell extracts were prepared at 36 hpi and western immunoblotted with (**a**) anti-Ebp1 or (**b**) anti-RPV. Cellular actin probed in parallel for equal loading (**c**). For measurement of viral titers, Ebp1-transfected cells were infected with RPV at an MOI of 1. "Mock" refers to cells transfected with pCAGGS alone (1 μg). At 96 h postinfection, virus titres were measured and plotted as a function of Ebp1 plasmid concentration (**d**). Titer values are the average of three independent experiments

Discussion

Viruses, being obligate intracellular pathogens, depend on many host factors for entry, transcription/replication, maturation and subsequent virus release. In non-segmented negative-sense RNA viruses, cytoskeletal proteins such as actin, tublin and profilin have been shown to be associated with viral RNA-dependent RNA polymerase (RdRp) and enhance virus transcription in vitro [5]. In addition, translation initiation factors eIF1α, β, and γ and heat shock

protein hsp72 have been shown to associate with viral RdRp and N proteins of VSV and CDV, respectively [17, 20].

In the present work, we provide evidence to show that Ebp1 protein affects rinderpest virus mRNA synthesis and virus yield. The data also show an antagonistic role for virus at the level of cellular Ebp1 transcription. Ebp1 acts as a negative regulator of influenza virus transcription and, subsequently, the virus yield in infected cells [12]. In addition, Ebp1 also gets upregulated in the initial phase of influenza virus infection. However, at the onset of PB1 synthesis, Ebp1 protein has been found to reach basal expression levels (A. Honda, unpublished). In RPV infection, Ebp1 is downregulated both at the transcriptional and translational levels. The mechanism by which the Ebp1 mRNA/protein level is downregulated in RPV-infected cells is still unclear. Cell death is a common phenomenon in paramyxovirus infection. However, the reduction in Ebp1 levels at 48 h postinfection is not due to cell death, since visible cell death commonly occurs around 72 h postinfection. In addition, endogenous mRNA levels of GAPDH, as measured by quantitative PCR analysis, did not change during infection.

Ebp1 is phosphorylated at both Ser 363 and Ser 261, and phosphorylation at these sites regulates its ability to suppress E2F-directed cell cycle-gene transcription as well as its intracellular degradation [2, 3, 14]. Ebp1 has also been shown to be ubiquitinated by a cellular protein BRE1, which is an E3 ligase for H2B mono-ubiquitination [15]. We speculate that virus infection may trigger the dephosphorylation of Ebp1 or BRE1-mediated ubiquitination of Ebp1, which leads to Ebp1 instability in the cell. The packaging of Ebp1 in the virion has been demonstrated in the present work. However, it is not clear why Ebp1, being a negative regulator of virus transcription, is packaged into the virions.

Ebp1 interacts with an array of cellular factors, such as Mybbpa, Pak1, Akt1, nucleoplasmin B23 and PKR, and modulates their activity to suppress cell division in cancer cell lines [1, 3, 14, 18, 21, 23]. In addition, Ebp1 associates with Rb, Sin3A and HDAC2 to repress E2F-regulated genes [25]. In the present study, the co-localization of Ebp1 with the viral RNP complex in cells and its packaging in purified virions suggested that Ebp1 interacts with RNP-associated viral protein/s or the N-RNA. Further, co-immunoprecipitation studies indicated that Ebp1 probably binds to viral N protein (or N-RNA). However, P could not be detected in the immunoprecipitate, suggesting that Ebp1 interaction with N protein (N-RNA) may likely eliminate P binding to assembled N protein. Alternatively, Ebp1 may interact with the nascent RNA itself [6]. The crystal structure of Ebp1 reveals the presence of a protein-interacting motif, ^{354}LKALL^{358}, and a lysine-rich motif, which offers a possibility of direct interaction between Ebp1 and viral proteins and/or genomic RNA [16]. The association of Ebp1 with mature ribosome and bcl-2 mRNP complex further provide evidence for its RNA-binding ability [6, 21].

Since Ebp1 is downregulated upon virus infection, it was of interest to test the effect of Ebp1 overexpression on viral transcription in vivo. Overexpression of Ebp1 at the time of viral infection results in decreased transcription and translation of viral proteins, which in turn affects the virus yield from infected cells. Similar results were observed with influenza virus, where Ebp1 was shown to affect viral transcription both in vivo and in vitro without affecting the cap binding and endonuclease activity of the viral polymerase [12]. However, rinderpest virus transcription takes place exclusively in the cytoplasm of infected cells. Ebp1-mediated inhibition may selectively target genome replication and/or the secondary transcription that occurs once viral protein synthesis begins through its interaction with N/N-RNA. Alternatively, Ebp1 may also affect other cellular factors that are crucial for viral transcription/replication in vivo. One candidate for this hypothesis is Akt, which was recently shown to play a key role in negative-sense RNA virus replication in vivo [22]. Knockdown of Akt in cells leads to decreased viral replication, and Akt was shown to phosphorylate HPIV3 P protein in vitro [22]. Since Ebp1 is a negative regulator of Akt signaling [10, 26], it is conceivable that, Ebp1 may indirectly regulate viral transcription/replication by inhibiting Akt activation in vivo. Ebp1-mediated inhibition of influenza virus transcription as well as RPV virus transcription, as shown in the present work, leads us to speculate that Ebp1 may function as an inhibitor for negative-sense RNA virus replication. However, further studies are needed to confirm this hypothesis in other viral systems.

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