Divergent Requirement for a DNA Repair Enzyme during Enterovirus Infections

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ABSTRACT Viruses of the Enterovirus genus of picornaviruses, including poliovirus, coxsackievirus B3 (CVB3), and human rhinovirus, commandeer the functions of host cell proteins to aid in the replication of their small viral genomic RNAs during infection. One of these host proteins is a cellular DNA repair enzyme known as 5′ tyrosyl-DNA phosphodiesterase 2 (TDP2). TDP2 was previously demonstrated to mediate the cleavage of a unique covalent linkage between a viral protein (VPg) and the 5′ end of picornavirus RNAs. Although VPg is absent from actively translating poliovirus mRNAs, the removal of VPg is not required for the in vitro translation and replication of the RNA. However, TDP2 appears to be excluded from replication and encapsidation sites during peak times of poliovirus infection of HeLa cells, suggesting a role for TDP2 during the viral replication cycle. Using a mouse embryonic fibroblast cell line lacking TDP2, we found that TDP2 is differentially required among enteroviruses. Our single-cycle viral growth analysis shows that CVB3 replication has a greater dependency on TDP2 than does poliovirus or human rhinovirus replication. During infection, CVB3 protein accumulation is undetectable (by Western blot analysis) in the absence of TDP2, whereas poliovirus protein accumulation is reduced but still detectable. Using an infectious CVB3 RNA with a reporter, CVB3 RNA could still be replicated in the absence of TDP2 following transfection, albeit at reduced levels. Overall, these results indicate that TDP2 potentiates viral replication during enterovirus infections of cultured cells, making TDP2 a potential target for antiviral development for picornovirus infections.

IMPORTANT Picornaviruses are one of the most prevalent groups of viruses that infect humans and livestock worldwide. These viruses include the human pathogens belonging to the Enterovirus genus, such as poliovirus, coxsackievirus B3 (CVB3), and human rhinovirus. Diseases caused by enteroviruses pose a major problem for public health and have significant economic impact. Poliovirus can cause paralytic poliomyelitis. CVB3 can cause hand, foot, and mouth disease and myocarditis. Human rhinovirus is the causative agent of the common cold, which has a severe economic impact due to lost productivity and severe health consequences in individuals with respiratory dysfunction, such as asthma. By gaining a better understanding of the enterovirus replication cycle, antiviral drugs against enteroviruses may be developed. Here, we report that the absence of the cellular enzyme TDP2 can significantly decrease viral yields of poliovirus, CVB3, and human rhinovirus, making TDP2 a potential target for an antiviral against enterovirus infections.

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The Picornviridae family includes human pathogens that are among the most common causes of viral illnesses in the world (1). This family includes viruses such as poliovirus, human rhinovirus (HRV), and coxsackievirus, all belonging to the Enterovirus genus. Poliomyelitis, the common cold, and myocarditis are examples of some of the diseases caused by these enteroviruses. Following cellular entry and virion uncoating, the small, positive-sense enterovirus RNA genome (~7.5 kDa) is translated and subsequently used as a template for viral RNA synthesis in the cytoplasm of infected cells. Since the genomic RNA encodes a limited number of viral proteins, enteroviruses must use host cell proteins in addition to their viral proteins to replicate in infected cells. This is a crucial element of the replication cycles carried out by all picornaviruses, including enteroviruses. To date, a number of host cell RNA binding proteins have been reported to play important roles in enterovirus translation initiation, RNA replication, or both (2–6). Enteroviruses can also alter host cell proteins to inhibit antiviral response pathways and stress granule formation. For example, the type I interferon response is inhibited by the degradation of the upstream cellular pathway regulators RIG-I, MDA5, and MAVS, via the activities of enterovirus proteinases 2A and 3C (reviewed in reference 7). These viral protein-
ases are encoded by the viral RNA genome, which consists of a highly structured 5′ noncoding region (NCR) followed by a single open reading frame (ORF). The ORF codes for the structural proteins and the nonstructural proteins (including the RNA-dependent RNA polymerase, 3D\textsuperscript{pol}). The coding region is followed by the 3′ NCR and the genetically encoded poly(A) tail. An overview of the enterovirus RNA genome is shown in Fig. 1.

In contrast to cellular mRNAs, the viral RNA genome lacks a 7-methylguanosine cap at the 5′ end, requiring enteroviruses to initiate cap-independent translation of its polyprotein via an internal ribosome entry site (IRES) in the 5′ NCR. Instead of a cap, picornavirus genomes possess a viral protein that is 20 to 22 amino acids in length (depending on the picornavirus) known as VPg (8, 9). VPg is covalently linked to the 5′ terminus of viral RNA via an O\textsuperscript{4}-(5′-uridylyl)tyrosine-phosphodiester bond as a result of viral RNA synthesis (Fig. 1) (10, 11). Since the viral RNA polymerase (3D\textsuperscript{pol}) cannot initiate viral RNA synthesis de novo, picornviruses have evolved to use a uridylylated VPg as a protein primer for the initiation of viral RNA synthesis (12). Early work had suggested that VPg is cleaved from the 5′ end of the genome to allow for polysome association and translation to occur but remains attached to the negative-strand RNA of the double-stranded replicative form (RF), as well as the positive strand of the replicative intermediate (RI), and newly synthesized virion RNA (vRNA) (13–19). It has also been reported that VPg can be detected on the 5′ end of the positive-strand RNA of RF molecules (20). Newly synthesized vRNAs either are then encapsidated into progeny virions or undergo additional rounds of translation and RNA replication.

In 1978, it was first discovered that a cellular activity, referred to as VPg unlinkase, cleaved the phosphodiester bond between VPg and viral RNA (21). VPg unlinkase activity was found to be present in both the nucleus and the cytoplasm of uninfected HeLa cells (22). The activity of the protein was shown to be Mg\textsuperscript{2+} or Mn\textsuperscript{2+} dependent but was inhibited in the presence of vanadate, SDS, Zn\textsuperscript{2+}, and EDTA, all of which are hallmarks of an enzyme (21, 23). More than 3 decades later, VPg unlinkase activity was shown to be mediated by the cellular DNA repair enzyme 5′ tyrosyl-DNA phosphodiesterase 2 (TDP2) (24). TDP2, also known as ITRAP and EAPII (25), is a predominantly nuclear enzyme, although it is present in the cytoplasm of the cell. As a DNA repair enzyme, TDP2 hydrolyzes the 5′ tyrosine-phosphodiester bond of single-stranded DNA in topoisomerase-mediated double-stranded DNA breaks (26). In addition to its DNA repair role, TDP2 has multiple roles in the uninfected cell, such as transcriptional regulation and signal transduction, through its multiple cellular binding partners, including ETS1, TRAFs, and CD40 (25, 27, 28). Additionally, TDP2 has been shown to have functional roles during other viral infections, such as those with human immunodeficiency virus, human papillomavirus, and hepatitis B virus (29–31).

During poliovirus infection, TDP2 relocates from the nucleus to the cytoplasm of the cell (24), most likely due to the alteration of nucleocytoplasmic trafficking that occurs during the course of picornavirus infections. This alteration leads to an increased concentration of a number of nucleus-resident proteins within the cytoplasm, some of which have been shown to be used for viral translation or RNA replication (reviewed in reference 6). Following its cytoplasmic accumulation, TDP2 appears to be excluded from putative sites of RNA replication and genome packaging during peak times of poliovirus infection. Significantly, the levels of VPg unlinkase activity remain unchanged in crude cytoplasmic extracts harvested throughout the course of poliovirus infection. Interestingly, that TDP2 activity is modulated by its cytoplasmic location or transient interactions with host or viral gene products (24, 32). This modulation may be required to ensure that, early during enterovirus infections, viral RNAs destined for translation do not have VPg linked to their 5′ ends while, at late times during infection, TDP2/VPg unlinkase is sequestered away from progeny RNAs to allow them to maintain VPg on their 5′ ends. The latter scenario appears to be a requirement for progeny virion formation, since only VPg-linked viral RNAs are packaged. However, previous studies have shown that VPg-linked viral RNA can form a translation initiation complex in vitro and that VPg-linked RNA can be translated and replicated in vitro (33, 34). Since the VPg unlinkase activity of TDP2 is not absolutely required for viral translation and RNA replication, its regulation during viral infection suggests a distinct function in coordinating the fate of cytoplasmic viral RNAs. We hypothesize that the role of TDP2 during enterovirus infections is to unlink VPg to distinguish viral RNA for use in translation, RNA synthesis, or encapsidation.

In this paper, we report that TDP2 is differentially required among enteroviruses. To determine the role of TDP2 during enterovirus infections, we took advantage of a mouse embryonic fibroblast (MEF) cell line lacking TDP2 (MEF-TDP2\textsuperscript{−/−}) (35).
We initially verified that the MEF-TDP2\(^{-/-}\) cell line lacked Vpg unlinkase activity using our rapid in vitro Vpg unlinkase assay (32). To determine if TDP2 is required for enterovirus replication, we determined the growth kinetics of poliovirus, coxsackievirus B3 (CVB3), and HRV during infections in the absence of TDP2. We found that TDP2 is divergently required for efficient enterovirus infections. High-level growth of CVB3 was exquisitely dependent on TDP2 expression, while levels of poliovirus and HRV replication were reduced by 1 to 2 orders of magnitude in the absence of TDP2. Western blot analysis confirmed that viral protein accumulation was greatly reduced in lysates from poliovirus-infected cells lacking TDP2 and was not detectable in lysates from CVB3-infected cells lacking TDP2. Although we found that CVB3 replication and protein accumulation following infection were not detectable by plaque assay and Western blot analysis, respectively, in the absence of TDP2, detectable (albeit highly reduced) levels of CVB3 replication were observed following transfection of an infectious CVB3 RNA encoding a reporter protein. Taken together, our data show that TDP2 activity potentiates enterovirus infections and, in the case of CVB3, is a required host function following virus infection of mouse cells.

RESULTS

Mouse embryonic fibroblasts genetically ablated for TDP2 lack VPG unlinkase activity. To determine if TDP2 is required during enterovirus infections, we measured picornavirus growth kinetics in the absence of TDP2. Since cells depleted of TDP2 through RNA interference (RNAi) may still result in low levels of Vpg unlinkase activity, we utilized a mouse embryonic fibroblast (MEF) cell line genetically ablated for TDP2 (MEF-TDP2\(^{-/-}\)) (35). To verify that the wild-type MEF cells (MEF-TDP2\(^{+/+}\)) expressed VPG unlinkase activity and that the MEF-TDP2\(^{-/-}\) cells lacked VPG unlinkase activity, we utilized a rapid in vitro VPG unlinkase assay previously described by Rozovics et al. (32). This assay allows for the visualization of linked versus unlinked VPG from a \(^{35}\)S)methionine-radiolabeled virion RNA (vRNA) substrate by utilizing a source of VPG unlinkase activity. \(^{35}\)S)methionine-radiolabeled substrate was generated by radiolabeling cells infected with a mutated poliovirus (W1-Vpg31) containing two methionines in the VPG sequence (36). The products of unlinkase reactions were resolved on a high-percent polyacrylamide gel and visualized by autoradiography. Since the full-length VPG-RNA encoding a reporter protein was not detectable by plaque assay and Western blot analysis, respectively, in the absence of TDP2, detectable (albeit highly reduced) levels of CVB3 replication were observed following transfection of an infectious CVB3 RNA encoding a reporter protein. Taken together, our data show that TDP2 activity potentiates enterovirus infections and, in the case of CVB3, is a required host function following virus infection of mouse cells.

TDP2 is required for efficient poliovirus replication following transfection of poliovirus vRNA in mouse embryonic fibroblasts. A previous study demonstrated that TDP2 not only hydrolyzes the phosphotyrosyl linkage between VPG and the 5' end of the poliovirus RNA but also relocalizes from the nucleus to the cytoplasm following poliovirus infection, suggesting that TDP2 is used during poliovirus replication (24). Additionally, this same study showed that TDP2 is relocalized to the cell periphery in foci distinct from putative viral replication and encapsidation sites during peak times of poliovirus replication, suggesting that TDP2 activity is modulated during the course of poliovirus infection (24). However, a recent report showed that removal of VPG from the 5' end of enterovirus RNA is not required for in vitro translation and RNA replication (34). To determine if TDP2 activity is required for replication of the prototypic enterovirus, poliovirus, we characterized the viral growth kinetics and quantified the viral yields of poliovirus-infected MEF cells lacking TDP2. Since mouse cells do not express the poliovirus receptor (PVR), they are naturally nonsusceptible to poliovirus infection. To bypass this limitation, we purified poliovirus vRNA (VPG-RNA) and transfected the vRNA into HeLa cells, MEF-TDP2\(^{+/+}\) cells, and MEF-TDP2\(^{-/-}\) cells. Poliovirus vRNA was transfected into HeLa cells as a positive control to measure maximum viral yields. We quantified viral yields by plaque assay and plotted titers (PFU per cell) on a logarithmic scale. Poliovirus replicated to approximately 2-log\(_{10}\) lower titers in MEF-TDP2\(^{+/+}\) cells than in HeLa cells, showing that poliovirus replicates less efficiently in this mouse cell line than in HeLa cells (Fig. 3). Significantly, poliovirus yields in mouse cells are reduced by up to 2 log\(_{10}\) units in the absence of TDP2. These results demonstrate that TDP2 is necessary for efficient poliovirus replication following poliovirus vRNA transfection into MEFs.

TDP2 is required for efficient poliovirus replication in mouse embryonic fibroblasts stably expressing the human poliovirus receptor. Although poliovirus yields were significantly reduced in the absence of TDP2 following vRNA transfection,
there are experimental caveats that may contribute to reduced viral yields. In particular, transfection of viral RNA bypasses normal cellular receptor-mediated entry pathways. It is likely that the uncoating of the vRNA following a normal infection determines the cytoplasmic delivery site of the vRNA and which end of the vRNA is initially exposed to the cytoplasm (37). To circumvent this potential limitation, we generated MEF-TDP2^+/+ and MEF-TDP2^−/− cell lines stably expressing the human poliovirus receptor (PVR) (38, 39) under blasticidin selection. Due to ambiguous results when trying to verify PVR protein expression in the stable cell line stably expressing the human PVR were similar in poliovirus susceptibility demonstrated. Accumulation of viral proteins 3A and 3AB was rescued in the MEF-TDP2^−/− cell line stably coexpressing PVR and hTDP2 (Fig. 5). In agreement with our single-cycle viral growth analysis, viral protein accumulation in the MEF-TDP2^−/− cell line coexpressing low levels of PVR mRNA, poliovirus replicates in the two additional MEF-TDP2^−/− stable cell lines expressing medium and high levels of PVR mRNA, albeit with a significant (1- to 2-log) reduction in viral yields compared to the MEF-TDP2^+/+ cells expressing PVR. The 0.5-log increase observed over the time course in the MEF-TDP2^−/− cell line expressing low levels of PVR mRNA does not represent a delay in poliovirus growth kinetics since we confirmed no increase in viral yields at 24 h postinfection (hpi) (data not shown). To confirm that the reduced viral titers observed were due to the absence of TDP2, we quantified the viral yields from poliovirus-infected MEF-TDP2^−/− cells coexpressing PVR and hTDP2 and found that poliovirus yields could be rescued by hTDP2 expression (Fig. 4B). Together, these results demonstrate that although virus yields are affected by the levels of PVR mRNA in transformed MEF cells, TDP2 is required for efficient poliovirus replication in mammalian cells.

To determine if TDP2 has an impact on poliovirus translation or RNA replication, we analyzed viral protein accumulation in the presence or absence of TDP2. We performed Western blot analysis using an antibody to poliovirus nonstructural protein 3A and its precursor 3AB, which are involved in poliovirus RNA replication (41, 42) (Fig. 5). In agreement with our single-cycle viral growth analysis, 3A and 3AB accumulation was rescued in the MEF-TDP2^+/+ cells expressing PVR compared to the MEF-TDP2^−/− cell line stably expressing PVR (see Fig. S2A, lanes 4 and 6). However, a previous report demonstrated that two different transgenic mouse lines expressing the human PVR were similar in poliovirus susceptibility despite differences in PVR RNA and protein expression (40). MEF-TDP2^+/+ and MEF-TDP2^−/− stable cell lines with PVR were infected with poliovirus, and viral yields were quantified by plaque assay. We found that poliovirus replicates somewhat less efficiently in the MEF-TDP2^+/+ cells expressing PVR than in HeLa cells (see Fig. S2B). Interestingly, poliovirus did not replicate in the MEF-TDP2^−/− cell line stably expressing PVR (see Fig. S2B), suggesting that TDP2 is required for productive poliovirus infection. To verify that the MEF-TDP2^−/− stable cell line was fully susceptible to poliovirus infection, we infected the MEF stable cell lines with a 5-fold increase in multiplicity of infection (MOI) and quantified the viral yields by plaque assay (see Fig. S2C). Similar results were observed at an MOI of 100 as at an MOI of 20.

Although a previous report indicated that variable PVR mRNA and protein expression does not greatly affect poliovirus susceptibility (40), we wanted to further verify that the phenotype that we observed in Fig. S2B and C in the supplemental material was due to the absence of TDP2 and not to low levels of PVR. We generated two additional MEF-TDP2^−/− cell lines stably expressing higher levels of PVR mRNA than our original transformants, referred to as medium- and high-PVR-expressing cell lines based upon the calculated band intensity of PVR mRNA on ethidium-stained agarose gels following RT-PCR (Fig. 4A, lanes 7 and 8). We also generated a MEF-TDP2^−/− stable cell line coexpressing PVR and human TDP2 (hTDP2) (25) to verify that the phenotype that we observed in the MEF-TDP2^−/− cell lines was due to the absence of TDP2 and not some secondary effect from gene ablation (Fig. 4A, lanes 9). TDP2 expression was verified in the MEF-TDP2^−/− stable cell line coexpressing PVR and hTDP2 by RT-PCR analysis (Fig. 4A, lane 9). Additionally, mouse TDP2 was confirmed to be absent from the MEF-TDP2^−/− stable cell lines (Fig. 4A).

To determine if TDP2 is required for poliovirus infection, each of the cell lines described above was infected with poliovirus and viral yields were quantified by plaque assay (Fig. 4B). Similarly to the previous single-cycle growth analysis, poliovirus growth kinetics were reduced in the MEF-TDP2^−/− cells expressing PVR compared to HeLa cells. In contrast to the previous single-cycle viral growth analysis of poliovirus in the MEF-TDP2^−/− cell line expressing low levels of PVR mRNA, poliovirus replicates in the two additional MEF-TDP2^−/− stable cell lines expressing medium and high levels of PVR mRNA, albeit with a significant (1- to 2-log) reduction in viral yields compared to the MEF-TDP2^+/+ cells expressing PVR. The 0.5-log increase observed over the time course in the MEF-TDP2^−/− cell line expressing low levels of PVR mRNA does not represent a delay in poliovirus growth kinetics since we confirmed no increase in viral yields at 24 h postinfection (hpi) (data not shown). To confirm that the reduced viral titers observed were due to the absence of TDP2, we quantified the viral yields from poliovirus-infected MEF-TDP2^−/− cells coexpressing PVR and hTDP2 and found that poliovirus yields could be rescued by hTDP2 expression (Fig. 4B). Together, these results demonstrate that although virus yields are affected by the levels of PVR mRNA in transformed MEF cells, TDP2 is required for efficient poliovirus replication in mammalian cells.
TDP2, just as viral yields are markedly reduced in the absence of TDP2.

**CVB3 replication is severely impaired in the absence of TDP2 in mouse embryonic fibroblasts.** We next determined if TDP2 was required during infection by the closely related enterovirus coxsackievirus B3 (CVB3). Since mice are naturally susceptible to CVB3 infection because they express the coxsackievirus adenovirus receptor (CAR), we infected MEF-TDP2+/+ and MEF-TDP2−/− cell monolayers with CVB3. We found that the viral growth was delayed and reduced in the MEF-TDP2+/+ cells compared to HeLa cells (Fig. 6A). Significantly, viral yields did not increase in the absence of TDP2 over the 16-h time course, while CVB3 increased up to 2 log10 in the MEF-TDP2+/+ cells, showing that TDP2 is essential for CVB3 replication (Fig. 6A).

To determine the effect of TDP2 on viral translation and RNA synthesis, we analyzed viral protein production in the MEF-TDP2+/+ and MEF-TDP2−/− cells by Western blot analysis using an antibody to the CVB3 nonstructural protein 3A and its precursor 3AB. We found that 3A and 3AB accumulation is reduced in an antibody to the CVB3 nonstructural protein 3A and its precursor 3AB. We found that 3A and 3AB accumulation is reduced in the absence of TDP2 over the 16-h time course, while CVB3 increased up to 2 log10 in the MEF-TDP2+/+ cells, showing that TDP2 is essential for CVB3 replication (Fig. 6A).

**FIG 4** TDP2 is used for efficient poliovirus replication following infection of mouse embryonic fibroblasts stably expressing the poliovirus receptor. (A) Human PVR and human and mouse TDP2 mRNA expression in the MEF-TDP2+/+ and MEF-TDP2−/− stable cell lines was verified by reverse transcription-PCR (RT-PCR) analysis. PCR products were separated by electrophoresis on an ethidium bromide-stained 1% agarose gel. A longer exposure of the gel depicting human PVR band intensity was quantified by Quantity One (Bio-Rad). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (B) Single-cycle growth analysis was carried out in HeLa cells or MEF-TDP2+/+ or MEF-TDP2−/− stable cell line monolayers following poliovirus infection at an MOI of 20. Cells and supernatant were collected every 2 h up to 12 h postinfection. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviations of the results from triplicate plaque assays. An asterisk (*) (Student’s t test; P < 0.05) or a double asterisk (**) (Student’s t test; P < 0.01) indicates statistical significance between MEF-TDP2+/+ and MEF-TDP2−/− stable cell lines.

**FIG 5** Poliovirus protein accumulation is reduced in the absence of TDP2. HeLa cell monolayers and MEF-TDP2+/+ and MEF-TDP2−/− stable cell line monolayers were mock or poliovirus infected at an MOI of 20. Cells and supernatant were collected at 0 or 8 h postinfection and used to generate NP-40 lysates. NP-40 lysate protein concentration was determined by Bradford assay. Mock- or poliovirus-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using anti-poliovirus 3A antibody or anti-PKM2 antibody (loading control) to visualize proteins.
in HeLa cells (Fig. 6B, lanes 3 and 6). In agreement with our single-cycle growth analysis of CVB3 in the MEF-TDP2−/− cell line, we found that 3A and 3AB do not accumulate to detectable levels in the presence of TDP2 (Fig. 6C). Since the transfected RNA was synthesized in vitro via a T7 promoter element, it lacks an authentic VPg linkage to the 5' end of the RNA, and we would therefore predict that transfected RNA would be translated in a TDP2-independent manner. Although still detectable, in the absence of the viral RNA synthesis inhibitor GuHCl, viral RNA replication was reduced in the MEF-TDP2−/− cell line at 8 and 12 h posttransfection (hpt) compared to the wild-type MEF cell line (Fig. 6C). Since viral RNA replication occurs in the absence of TDP2 following CVB3 RNA transfection, we wanted to verify that CVB3 replication was not merely delayed in the absence of TDP2. We quantified viral yields in CVB3-infected MEF-TDP2+/+ and MEF-TDP2−/− cells at 24

FIG 6 TDP2 is required for CVB3 infection in mouse embryonic fibroblasts. (A) Single-cycle growth analysis was carried out in HeLa cells and MEF-TDP2+/+ and MEF-TDP2−/− monolayers following infection with CVB3 at an MOI of 20. Cells and supernatant were collected every 2 h up to 16 h postinfection (hpi). Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviations of the results from triplicate plaque assays. An asterisk (*) (Student’s t test; \( P < 0.05 \)) or a double asterisk (***) (Student’s t test; \( P < 0.01 \)) indicates statistical significance between MEF-TDP2+/+ and MEF-TDP2−/− data points. (B) HeLa cells and MEF-TDP2+/+ and MEF-TDP2−/− cell monolayers were mock or CVB3 infected at an MOI of 20. Cells were harvested at 0 or 8 hpi and used to generate NP-40 lysates. NP-40 lysate protein concentration was determined by Bradford assay. Mock- or CVB3-infected NP-40 lysate was subjected to SDS-PAGE and Western blot analysis using an anti-CVB3 3A antibody or anti-PKM2 antibody (loading control) to visualize proteins. The image in panel B was cropped due to an absence of TDP2 (Fig. 6B, lane 9). Since Western blot analysis may not be sensitive enough to detect low levels of viral protein expression over a 12-h time course. As expected, in the presence of GuHCl, viral RNA was translated in the presence or absence of TDP2 (Fig. 6C). Since the transfected RNA was synthesized in vitro via a T7 promoter element, it lacks an authentic VPg linkage to the 5' end of the RNA, and we would therefore predict that transfected RNA would be translated in a TDP2-independent manner. Although still detectable, in the absence of the viral RNA synthesis inhibitor GuHCl, viral RNA replication was reduced in the MEF-TDP2−/− cell line at 8 and 12 h posttransfection (hpt) compared to the wild-type MEF cell line (Fig. 6C). Since viral RNA replication occurs in the absence of TDP2 following CVB3 RNA transfection, we wanted to verify that CVB3 replication was not merely delayed in the absence of TDP2. We quantified viral yields in CVB3-infected MEF-TDP2+/+ and MEF-TDP2−/− cells at 24
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FIG 7  TDP2 is used for efficient rhinovirus replication in mouse embryonic fibroblasts. Single-cycle growth analysis was carried out in MEF-TDP2+/+ and MEF-TDP2−/− monolayers following infection with the minor group A human rhinovirus (HRV1a) at an MOI of 20. Cells and supernatant were collected every 5 h up to 30 h postinfection. Virus yields (PFU) were quantified by plaque assays performed on HeLa cell monolayers and divided by the total cell count prior to infection (PFU/cell). Viral yields were plotted on a logarithmic scale. The error bars indicate standard deviations of the results from triplicate plaque assays. An asterisk (*) (Student’s t test; P < 0.05) or a double asterisk (**) (Student’s t test; P < 0.01) indicates statistical significance between MEF-TDP2+/+ and MEF-TDP2−/− data points.

hpi and found that viral yields did not increase at 24 hpi in the absence of TDP2. These data show that CVB3 replication was not delayed in the MEF-TDP2−/− cell line (Fig. 6D). Collectively, our results show that CVB3 RNA can be translated and replicated in the absence of TDP2 following transfection of CVB3 RNA; however, viral production is dramatically impaired in the absence of TDP2. Furthermore, reduced replication in the MEF-TDP2−/− cells does not delay the onset of CVB3 replication.

Human rhinovirus uses TDP2 in mouse embryonic fibroblasts for efficient viral replication. Finally, we determined if TDP2 was required for the replication of another picornavirus classified as an enterovirus, human rhinovirus (HRV). Since mouse embryonic fibroblasts lack the major HRV group receptor, ICAM-1, but express the minor HRV group receptor, low-density lipoprotein receptor (LDLR), we infected the MEF-TDP2+/+ cell line and the TDP2−/− cell line with the minor rhinovirus group A member HRV1a. We observed that viral yields were reduced by slightly more than 1 log10 in the absence of TDP2 at peak viral titers (Fig. 7). These results show that TDP2 potentiates HRV1a replication in mouse embryonic fibroblasts; however, this effect is somewhat reduced compared to what we observed for poliovirus and CVB3 replication.

DISCUSSION
In this study, we showed that the VPg-unlinking activity of host cell DNA repair enzyme TDP2 is diversely utilized during the course of enterovirus infections. This cellular activity was shown to hydrolyze the phosphotyrosyl linkage between the small viral protein VPg and the 5′ end of poliovirus RNA (21). Before the identification of VPg unlinkase as TDP2 (24), VPg unlinkase was characterized as having hallmarks of a bona fide enzyme, its activity unaffected by the presence of exogenous VPg, and if assayed in crude extracts, its unlinking activity was unchanged over the course of poliovirus infection in HeLa cells (22, 32). However, following the identification of TDP2 as VPg unlinkase, TDP2 was shown to relocalize to the cell periphery in areas distinct from putative viral replication and encapsidation sites during peak times of poliovirus infection (24). This relocalization pattern at peak times of infection suggests that TDP2 activity is used early on during infection, in agreement with previous studies showing that VPg is absent from ribosome-associated viral RNA (13, 17–19). Collectively, these previous findings suggest that TDP2 activity is modulated during the course of poliovirus infection. It is possible that the relocalization of TDP2 to the cell periphery at peak times of infection is a regulatory mechanism used by the virus to avoid premature removal of VPg from newly synthesized viral RNAs destined to become encapsidated. Although the functional role of TDP2 during the course of enterovirus infections remains unknown, we hypothesized that TDP2/VPg unlinkase activity or the subsequent masking of that activity marks viral RNA as a template for viral translation, RNA synthesis, or encapsidation.

Our findings provide evidence that TDP2 activity is required for efficient poliovirus replication. We found that a mouse embryonic fibroblast cell line genetically ablated for TDP2 lacks VPg unlinkase activity, using a previously described in vitro VPg unlinkase assay (32). Although it is possible that our in vitro assay is not sensitive enough to detect low levels of VPg unlinkase activity in the MEF cells lacking TDP2 due to another source of VPg unlinkase activity, previous studies from our laboratory showed that another phosphodiesterase that hydrolyzes 3′ phosphotyrosyl bonds (3′ tyrosyl-DNA phosphodiesterase 1 [TDP1]) lacks VPg unlinkase activity (32). Using the mouse cell line lacking VPg unlinkase activity, we first examined the effect of TDP2 during the course of infection of the prototypic enterovirus, poliovirus. We used RNA transfections of mouse cells lacking the gene for TDP2 or infections using these same cells stably transformed with a cDNA expressing the human poliovirus receptor. In both cases, viral yields were reduced by approximately 90 to 99% in the absence of TDP2. Importantly, the decrease in poliovirus yields observed in the cell lines lacking TDP2 but stably expressing high levels of PVR was verified to be due to the absence of TDP2, because poliovirus yields could be rescued in the mouse cells stably coexpressing PVR and human TDP2.

We found that viral protein accumulation was severely reduced in the absence of TDP2, suggesting that TDP2 plays a role in poliovirus translation, RNA synthesis, or encapsidation, since these processes are tightly coupled during the course of infection (44, 45). In the absence of TDP2, poliovirus RNA can be translated and replicated, albeit at reduced levels. These findings agree with previous reports that show that VPg-linked poliovirus RNA can form a translation initiation complex in vitro and VPg-linked poliovirus RNA can be translated and replicated in vitro (33, 34). Thus, the activity of TDP2 does not appear to be directly involved in translation initiation or specific steps in viral RNA synthesis. It remains to be determined how the fate of viral RNA is altered if VPg remains linked to ribosome-associated viral RNA in the absence of TDP2. Considering that poliovirus can still replicate in the absence of TDP2, although with reduced efficiency, our results suggest that VPg could remain linked to ribosome-associated RNA in the absence of TDP2. The detection of ribosome-associated, linked RNA in the absence of TDP2 would indicate that another cellular protein has redundant VPg unlinkase activity.
In contrast to poliovirus, we found that the closely related enterovirus CVB3 does not appear to replicate in the absence of TDP2 in murine cells. Our single-cycle growth analysis and Western blotting assays confirmed that viral yields do not increase and viral proteins do not accumulate, respectively, in the absence of TDP2. Despite these results, CVB3 RNA can be translated and replicated at reduced levels. Although it is possible that the observed translation and replication of CVB3 RNA are due to the RNA being transfected versus infected, this seems unlikely since we found a similar dependency on TDP2 following RNA transfection or poliovirus infection of MEF cells. Additionally, our results agree with a previous study that shows that VPg unlinkase-resistant CVB3 VPg-RNA can undergo in vitro translation and replication (34). If CVB3 RNA can be translated and replicated in the absence of TDP2, why is there no increase in viral yields or viral protein accumulation? Although we are currently exploring the answer to this question, the greater dependence of CVB3 on TDP2 for VPg unlinkase activity compared to other enteroviruses could be related to CVB3-specific requirements for host factors involved in partitioning translation/replication complexes for early versus late stage steps in the viral life cycle. The putative interaction of TDP2 with such factors may be required to promote the assembly of progeny virions. It is also possible that CVB3 circumvents the mouse host immune response less efficiently than poliovirus. This weakened ability to counter the host immune response, in combination with reduced levels of CVB3 replication in the mouse embryonic fibroblasts, could lead to the phenotype observed in the MEF-TDP2−/− cells.

Previous reports showed that VPg unlinkase and recombinant TDP2 can hydrolyze the phosphotyrosyl linkage between human rhinovirus VPg and poliovirus RNA derived from a chimeric HRV-poliovirus (24, 32). Our results show that human rhinovirus yields are reduced up to 90% in the absence of TDP2. Similarly to poliovirus, rhinovirus can still replicate in the absence of TDP2, but with reduced efficiency. Collectively, our results show that enteroviruses have disparate dependencies on TDP2 for replication. We find that CVB3 requires TDP2 for any detectable replication in murine cells, whereas poliovirus and human rhinovirus have a less strict dependence on TDP2. The fact that all of the enteroviruses that we tested have some level of dependency on TDP2 is consistent with our hypothesis that TDP2 regulates, in part, enterovirus infections. Our proposed model in Fig. 8 shows that in the absence of TDP2, viral translation and RNA synthesis can still occur; however, nascent viral RNAs do not undergo additional rounds of translation but can instead become prematurely encapsidated. The premature encapsidation of newly synthesized viral RNAs leads to an overall decrease in overall levels of viral translation and viral RNA synthesis in infected cells, thus resulting in a decrease in viral yields. In addition, a decrease in viral translation would lead to a decrease in capsid protein production that is necessary for efficient encapsidation of newly synthesized viral RNAs.

Since the steps in viral replication are tightly coupled, the exact role that TDP2 plays during infection may prove difficult to dissect (44, 45). Understanding this role should be facilitated by first defining the mechanism that enteroviruses employ to modulate TDP2 activity. As previously noted, TDP2 has multiple binding partners involved in different aspects of cell signaling and transcriptional regulation. Since enteroviruses have been shown to modulate cellular proteins via their virally encoded proteinases, it is possible that enterovirus proteinases directly modify TDP2 or indirectly modify the activity of TDP2 by altering its binding partners. Although our results show that TDP2 potentiates enterovirus replication, it remains to be determined if TDP2 is required for other picornaviruses that differ from the closely related members of the enterovirus genus, such as cardioviruses and aphthoviruses. Since TDP2 is required for CVB3 replication and may be required for other picornavirus infections, a small-molecule inhibitor tar-

FIG 8 The presence or absence of VPg marks enterovirus RNAs for viral translation, RNA synthesis, or encapsidation. The proposed role of TDP2 during enterovirus infections is illustrated. (A) Following infection, enterovirus vRNA (VPg-RNA) is released into the cell cytoplasm. TDP2 (yellow "Pac-Man" symbol) hydrolyzes the phosphotyrosyl bond between VPg (red circle) and the 5′ end of the viral RNA. Next, the viral RNA undergoes translation and negative-strand RNA synthesis. The negative-strand viral RNA serves as a template for positive-strand viral RNA synthesis by 3Dpol (purple oval). It should be noted that previous studies showed VPg both linked and absent from the 5′ end of the positive-strand RNA of the RF (20). The newly synthesized positive-strand RNA (VPg-RNA) can either undergo additional rounds of viral translation and RNA replication or become encapsidated into progeny virions. (B) In the absence of TDP2, VPg-RNA can still undergo viral translation and negative-strand RNA synthesis, albeit at reduced levels, due to the presence of VPg on the viral RNA. Following positive-strand RNA synthesis, the newly synthesized viral RNA (VPg-RNA) is prematurely encapsidated, leading to reduced viral titers.
geting the VPg unlinkase activity of TDP2 could be developed to serve as a broad-spectrum antiviral. A previous study has identified toxoflavin, its derivatives, and deazaflavins as the first submicromolar, selective TDP2 inhibitors by high-throughput screening (46). Although further studies will have to be carried out to test the effects of small-molecule inhibitors on picornavirus infections, TDP2 is an attractive potential target for novel antiviral therapeutics.

MATERIALS AND METHODS

Cell culture and virus stocks. Wild-type TDP2 mouse embryonic fibroblast (MEF-TDP2+/+) and knockout TDP2 mouse embryonic fibroblast (MEF-TDP2−/−) cell lines were described by Zeng et al. (35). MEF-TDP2+/+ and MEF-TDP2−/− cells were grown as monolayers in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS). HeLa cells were grown as monolayers in DMEM supplemented with 8% newborn calf serum (NCS) or in suspension culture in suspension minimal essential medium (S-MEM) supplemented with 8% NCS or methionine-free DMEM.

The W1-VPg31 virus (36) was used to infect HeLa cells in suspension for the purification of poliovirus virion RNA (vRNA) substrate. Virus stock for HRV1a was kindly provided by Yury Bochkov at the University of Wisconsin, Madison. All virus stocks were produced by three serial passages in HeLa cells.

[35S]methionine-labeled poliovirus virion RNA substrate generation and VPg unlinkase assay. Radiolabeled poliovirus vRNA substrate was prepared from the W1-VPg31 virus (36) as previously described (32). In brief, HeLa cells were grown in suspension. Following infection with W1-VPg31 (MOI of 20), HeLa cells were starved of methionine for 2 h at 37°C. [35S]methionine (2.5 mcI) was added to the infected cell suspension and further incubated for 3 h. The radiolabeled, infected cells were pelleted and subjected to the previously described poliovirus vRNA purification scheme (32). The final volume of the radiolabeled substrate was adjusted to 1,000 cpm/µl (160 ng/µl).

The in vitro VPg unlinkase assay was carried out as previously described (24, 32). For our experiments, a 20-µl reaction mixture containing 1,000 cpm (160 ng) of radiolabeled poliovirus vRNA was incubated with RNase A (10 µg) or a source of VPg unlinkase activity in the presence of 2 mM MgCl₂ in unlinkase buffer (20 mM Tris-HCl buffer, pH 7.5, 1 mM diithiothreitol [DTT], 5% [vol/vol] glycerol) at 30°C for 30 min. The reactions were resolved by electrophoresis on an SDS-containing 13.5% polyacrylamide gel in Tris-Tricine for 2.5 h (for VPg unlinkase activity) or reactions were resolved by electrophoresis on an SDS-containing 12.5% polyacrylamide gel for HRV1a. Prior to electrophoresis, the VPg unlinkase products of the ligation reaction were transformed into DH10β competent Escherichia coli. pFlag-CMV-TDP2 was isolated with a miniprep (Qiagen), sequenced, and cesium chloride purified for transfection. The MEF-TDP2−/− stable cell lines coexpressing PVR and human TDP2 were generated as described above. Human TDP2 mRNA expression was verified by RT-PCR analysis using forward (5’-GACTGCTGGATGAGTTTTGAGGCA-3’) and reverse (5’-GCAAGATGGCGAATTTTGGGCA-3’) primers.

Infections were carried out as described above. Cells were incubated at 37°C. Cells and supernatant were collected and subjected to 5 freeze-thaw cycles. Viral yields were quantified by plaque assay on HeLa cell monolayers. Viral yields are reported as PFU per cell and plotted on a logarithmic scale. Plaque assays were performed in triplicate. Errors are reported as standard deviations.

Preparation of lysates from uninfected and infected cells and Western blot analysis. Infections were carried out as described above. Cells were harvested and pelleted at 0 and 8 h postinfection. Samples were washed with 1× PBS 3 times and the pellet was resuspended in NP-40 lysis buffer (50 mM Tris-HCl, pH 7.5, 5 mM EDTA, 150 mM NaCl, 1% NP-40) and incubated on ice for 30 min. Cell debris was pelleted, and the supernatant was collected. Protein concentration was measured by a Bradford assay. For Western blot analysis, 50 µg of NP-40 lysates from poliovirus-infected cells and 75 µg of NP-40 lysates from CVB3-infected cells were subjected to electrophoresis on SDS-containing 12.5% polyacrylamide gels. Proteins were then transferred to a polyvinylidene difluoride (PVDF) membrane. Antibodies to poliovirus protein 3A at a dilution of 1:2,000 or CVB3 protein 3A at a dilution of 1:5,000 were used to detect viral protein accumulation in the NP-40 lysates. The mouse monoclonal antibody to poliovirus protein 3A was kindly provided by George Below at the University of Maryland. The mouse monoclonal antibody to

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CVB3 protein 3A was kindly provided by J. Lindsay Whitton at the Scripps Research Institute, La Jolla, CA. Antibody PKM2 (Bethyl) at a dilution of 1:2,000 was used to detect endogenous PKM2 as a protein loading control. Protein bands were visualized by enhanced chemiluminescence (ECL) with Western blotting substrate (Life Technologies).

**In vitro transcription and transfection of RLuc-CVB3 RNA for mRNA translation and replication assays.** For generation of *in vitro* Renilla luciferase CVB3 (RLuc-CVB3) RNA transcripts, the infectious RLuc-CVB3 clone p53CB3/T-7-Rluc (43), kindly provided by Frank J. M. van Kuppeveld at the University of Utrecht, Utrecht, the Netherlands, was linearized with Sall to generate a transcription template. RNA transcription was performed using a MEGAscript T7 transcription kit (Ambion). RNA transcript was purified using the RNaseasy kit (Qiagen). HeLa, MEF, TDP2 +/−, and MEF−TDP2 −/− cell monolayers in 10-cm 2 plates were transfected with 2 μg of RNA transcript per plate using the TransIT-mRNA transfection kit (Mirus Bio). For the translation assay, transfection was carried out in the presence of 2.5 mM CuHCl, a viral RNA synthesis inhibitor. For the replication assay, transfection was carried out in parallel in the absence of CuHCl. Cells were washed with 1× PBS and lysed in 1× Renilla luciferase assay lysis buffer (Promega) for 15 min. RLuc values were measured using the Renilla luciferase assay system (Promega). Relative light units (RLU)/second/cell were calculated, and values were plotted as RLU/cell. Transfections were carried out in three separate biological replicates, with one of the replicates carried out as a technical duplicate. The RLuc values from the technical duplicate were averaged and used as a representation of a biological replicate. The RLuc values from the biological replicates were averaged, and the standard error of the mean was calculated for the error bars.

**SUPPLEMENTAL MATERIAL**

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01931-15/-/DCSupplemental.

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