An RNA virus hijacks an incognito function of a DNA repair enzyme

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A previously described mammalian cell activity, called VPg unlinkase, specifically cleaves a unique protein–RNA covalent linkage generated during the viral genomic RNA replication steps of a picornavirus infection. For over three decades, the identity of this cellular activity and its normal role in the uninfected cell had remained elusive. Here we report the purification and identification of VPg unlinkase as the DNA repair enzyme, 5′-tyrosyl–DNA phosphodiesterase-2 (TDP2). Our data show that VPg unlinkase activity in different mammalian cell lines correlates with their differential expression of TDP2. Furthermore, we show that recombinant TDP2 can cleave the protein–RNA linkage generated by different picornaviruses without impairing the integrity of viral RNA. Our results reveal a unique RNA repair-like function for TDP2 and suggest an unusual role in host–pathogen interactions for this cellular enzyme. On the basis of the identification of TDP2 as a potential antiviral target, our findings may lead to the development of universal therapeutics to treat the millions of individuals afflicted annually with diseases caused by picornaviruses, including myocarditis, aseptic meningitis, encephalitis, hepatitis, and the common cold.

5′-tyrosyl-RNA phosphodiesterase | poliovirus | human rhinovirus | translation initiation

RNA viruses with limited genomic coding capacities require the extensive use of host cell functions to carry out their replication cycles in mammalian cells. As such, unraveling the mechanisms of gene expression and replication for these viruses necessitates that we identify the cellular proteins responsible for such functions. For the positive-strand RNA viruses in the picornavirus family (including poliovirus, human rhinovirus, and foot-and-mouth disease virus), host cell functions are required for several steps in their intracellular replication cycles, including viral translation and RNA synthesis. These viruses use a small viral protein (VPg) as a primer for viral RNA synthesis, which results in the linkage of all nascent viral RNAs to VPg via an O⁴-(5′-uridylyl)tyrosine bond (reviewed in ref. 1). Following genome release from the infecting virion, however, the VPg–RNA linkage of virion RNA (vRNA) is short lived (refer to the picornavirus RNA species depicted in Fig. 1A). Upon polysome association, VPg is removed from vRNA by a cellular enzyme (2), which will be referred to as “VPg unlinkase” in this report. Interestingly, all viral RNAs packaged into virions maintain VPg at the 5′ ends of progeny RNAs, suggesting that VPg unlinkase activity may be modulated during the later stages of picornavirus infections. Since its discovery in 1978 (2), several studies have described the partial purification (3, 4) and biochemical characterization (reviewed in ref. 5) of VPg unlinkase and explored its possible role(s) during picornavirus infections (2, 3, 6–9). Despite these efforts, the cellular identity of this remarkable enzyme has remained elusive. In this report we describe the identification of VPg unlinkase from uninfected HeLa cells as 5′-tyrosyl–DNA phosphodiesterase-2 (TDP2), a DNA repair enzyme with additional proposed functions in the mammalian cell. Western blot analysis demonstrated the presence of TDP2 in fractionated extracts enriched for VPg unlinkase activity and correlated cell-specific differences in VPg unlinkase activity with levels of TDP2. Using recombinant TDP2

generated in bacteria, we showed the in vitro cleavage of the VPg–RNA linkage for both poliovirus and human rhinovirus. Finally, confocal microscopy of HeLa cells infected with poliovirus revealed that TDP2 is relocalized in the cell to cytoplasmic sites distinct from those containing viral proteins associated with RNA replication or encapsidation, suggesting that the virus may exclude VPg unlinkase/TDP2 from viral complexes that require an intact VPg–RNA linkage during the later stages of infection. We discuss the implications of our findings to highlight how a large family of medically important RNA viruses appears to have hijacked a unique phosphodiesterase function from a host cell DNA repair enzyme, providing an extraordinary example of pathogen ingenuity.

Results and Discussion

To identify VPg unlinkase, we developed a multi-step purification procedure using our recently described VPg unlinkase activity assay, which resolves [³⁵S]methionine radiolabeled-VPg released from poliovirus vRNA (³⁵S-PV1–RNA) by Tris-tricine SDS/PAGE (7). Our assay was optimized for rapid detection of VPg unlinkase activity (*Materials and Methods*). During the development of this purification scheme, we screened different synthetic compounds and nucleic acids as competitive inhibitors to be used in the affinity purification of VPg unlinkase. We made a fortuitous observation that single-strand DNA (ssDNA) is ~100-fold more efficient at inhibiting VPg unlinkase activity than synthetic RNA with or without a 5′-tyrosyl–RNA bond (Fig. S1). This key finding prompted us to include ssDNA cellulose in our purification protocol.

Because VPg unlinkase activity is found in both cytoplasmic and nuclear extracts (2), we initiated our purification procedure using total cell homogenate from uninfected HeLa cells. After subjecting the homogenate to high-speed centrifugation to pellet cellular debris and large complexes containing nucleic-acid-binding proteins, the supernatant (S370), which contained ~75% of the initial activity, was fractionated sequentially by heparin-sepharose, ssDNA-cellulose, anion-exchange, size-exclusion, and cation-exchange chromatography, resulting in the generation of a nearly homogeneous enzyme preparation in which activity was enriched by >10,000-fold (Fig. 1B and C). The resulting 38-kDa polypeptide (p38) isolated by this purification scheme (Fig. 1B, lane 7, purification step F) corresponded in size with the VPg unlinkase detected during our initial characterization of this activity (Fig. S2). Analysis of fractions from purification step F (cation-exchange chromatography) verified the coelution of p38 (Fig. 2A, Lower) with VPg unlinkase activity (Fig. 2A, Upper and Fig. S3).

The protein corresponding to p38 was excised from two different lanes of a polyacrylamide gel (Fig. 2A, lanes 7 and 8,

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Fig. 2. Identification of p38 as TDP2. (A) Quantified VPg unlinkase activity profile (*Upper*, red histogram) and SDS/PAGE analysis (*Lower*) of fractions generated by purification step F show the coelution of p38 with VPg unlinkase activity. Diagonal line (brown) indicates the linear gradient of increasing NaCl concentration (~150 to 350 mM) used to elute VPg unlinkase. (B) Mass spectrometry analysis of p38 isolated from lane 7 (p38-F12, *Upper*) and lane 8 (p38-F13, *Lower*) of the polyacrylamide gel shown in A, *Lower* identified several tryptic peptides corresponding to TDP2 (in red; overlapping sequence is underlined). (C) Western blot analysis using anti-TDP2 polyclonal antibody (Santa Cruz Biotechnology) confirms the isolation of TDP2 by our purification protocol (lanes labeled by purification step). (D) Relative TDP2 expression levels in different cellular extracts correlate with the VPg unlinkase activity detected previously (7): HeLa (human cervical carcinoma cell line) > K562 (human myeloid leukemia cell line) > NGP (human neuroblastoma cell line) > SKOV3 (human ovarian carcinoma cell line) > RRL (rabbit reticulocyte lysate).

protein 3A (Fig. 4A) and viral capsid proteins (Fig. 4B). At the periphery of the infected cell, TDP2, 3A, and capsid proteins were found in close proximity to one another; however, they did not

appear to colocalize (confirmed by z-stack analysis). From the changes in the subcellular localization of TDP2 that occur at later times postinfection, we propose that picornaviruses exclude

Fig. 3. Recombinant GST-TDP2 has authentic VPg unlinkase activity. (A) Equivalent amounts of partially purified VPg unlinkase and GST-TDP2 both unlink VPg from [³⁵S]VPg-PV RNA (Upper), without any apparent degradation of PV RNA (Lower). (B) Increasing amounts of partially purified VPg unlinkase and GST-TDP2, but not GST, unlink VPg from [³⁵S]VPg-PV RNA and [³⁵S]VPg HRV-PV RNA. Reactions containing RNase A were included to generate markers for VPg-pUp (lanes 6 and 13).

Fig. 4. Poliovirus infection relocates rDP2. (A) Immunofluorescence of TDP2 and viral RNA replication protein 3A. Mock- (Top) or poliovirus-infected HeLa cells were fixed at specific times postinfection; shown are images generated at 2 h (Middle) or 4 h (Bottom) postinfection. Cells were colabeled with antibodies directed against TDP2 (shown in red) or poliovirus protein 3A (green), and nuclei were stained with DAPI. Dashed arrows indicate regions that were largely devoid of TDP2. Arrows indicate regions of poliovirus 3A found adjacent to regions of TDP2. (B) Immunofluorescence of TDP2 and capsid proteins. Mock- (Top) or poliovirus-infected HeLa cells were prepared as described in A above, except that cells were colabeled with anti-TDP2 or poliovirus anti-capsid antibodies. Dashed arrows indicate regions that were largely devoid of TDP2. Arrows indicate regions of viral capsid proteins found adjacent to regions of TDP2.

TDP2 from RNA replication complexes to protect VPg-linked progeny RNAs required for encapsidation.

Although TDP2 is the only known 5'-tyrosyl-DNA phosphodiesterase found in vertebrate cells (13), it was initially disregarded as a putative VPg unlinkase candidate for several reasons. First, it has been reported that VPg unlinkase cannot cleave the tyrosyl-nucleic acid linkage of a synthetic 5'-tyrosyl-DNA substrate (14). However, in an effort to understand why VPg unlinkase is also unable to hydrolyze the serine-RNA linkage of the genome-linked protein of cowpea mosaic virus (15, 16), we considered the possibility that electrostatic interactions with tyrosine (linked to

genomic RNA) are important determinants for substrate recognition by VPg unlinkase, similar to the mechanisms used by apurinic/aprimidinic endonuclease (17), cap-binding proteins (18), and a wide range of other protein-ligand interactions (reviewed in ref. 19). This model predicts that the 3,5-[¹²⁵I]diiodotyrosine-labeled synthetic 5'-tyrosyl-DNA substrate used in the above-referenced work is incompatible with the active site of VPg unlinkase. Second, mass spectrometry analysis of fractions containing VPg unlinkase generated by previous purification protocols had not detected TDP2 (7). Considering that TDP2 is a fast (20) and low abundance enzyme (21), it is likely that protein purity

a 5'-tyrosyl-RNA phosphodiesterase activity. The mammalian enzyme capable of 3'-tyrosyl-DNA phosphodiesterase activity (Tdp1) harbors a limited 3'-nucleosidase activity capable of acting on both DNA and RNA substrates (28). However, to date this enzyme has not been reported to cleave tyrosyl-RNA linkages. The unique activity of TDP2 in hydrolyzing both tyrosyl-RNA and tyrosyl-DNA linkages may, in part, be a consequence of the similarities shared by DNA and RNA. It could also be a consequence of the evolution of biochemical pathways using TDP2-like enzymes for the repair or functional regulation of unique protein-nucleic acid species. The ability of TDP2 to hydrolyze viral protein-RNA linkages may be the remaining trace to an ancestral enzyme in the RNA world that, in the course of evolution, acquired the ability to cleave protein-DNA linkages and to mediate new functions (e.g., transcriptional regulation and signal transduction) via protein-protein interactions without disrupting the ancient RNA-specific phosphodiesterase activity.

Materials and Methods

VPg Unlinkase Assays, Purification, Detection, and Recombinant TDP2. Except for an initial 3-h methionine starvation during viral infection, generation of [³⁵S]methionine-labeled vRNA was performed as previously described (7). To generate the HeLa cell homogenate used for the isolation of VPg unlinkase, 5 mL of packed HeLa cells was homogenized by cryogenic grinding (Retsch) and then solubilized in PDEG10 buffer [20 mM phosphate buffer, pH 7.0, 5 mM DTT, 1 mM EDTA, 10% (vol/vol) glycerol]. The resulting extract was then subjected to the purification scheme described in the text. For the detection of VPg unlinkase activity, 2-μL aliquots of samples were incubated with 700 cpm [³⁵S]methionine-labeled vRNA (~0.15 pmol) in a 20-μL reaction volume containing PDEG10 buffer supplemented with 4 mM MgCl₂ at 30 °C for 3 min (enzyme detection) or 30 min (to detect nuclease activity). An additional 1 μg PV1 vRNA was included for visualization of reactions by 1% (wt/vol) agarose electrophoresis. Reactions were either subjected to 13.5% (wt/vol) Tris-tricine PAGE for 2 h (for fast detection of activity) or 16 h (to resolve VPg from VPg-pUp) and quantified as described previously (7). Anti-

TDP2 antibodies were purchased from Santa Cruz Biotechnology (anti-EAII; sc-135214) or Bethyl Laboratories (anti-TDP2). Monoclonal antibody to poliovirus protein 3A was a generous gift from George Belov, University of Maryland, College Park, MD. The plasmid construct expressing GST-TDP2 (21) was a generous gift from Runzhao Li, Emory University, Atlanta. Recombinant GST-TDP2 was expressed and purified on glutathione-agarose according to the manufacturer's protocol (GE Healthcare).

Confocal Microscopy. For immunofluorescence, HeLa cells were grown on coverslips and mock infected or infected with poliovirus at a multiplicity of infection (MOI) of 20. Cells were washed and fixed at 1–5 h postinfection (every hour) in 4% (vol/vol) formaldehyde. Cell membranes were permeabilized with 0.5% (vol/vol) Nonidet P-40, and cells were colabeled with rabbit anti-TDP2 (Bethyl Laboratories) and either poliovirus mouse anti-3A or mouse anticapsid (Millipore). Secondary antibodies (goat antirabbit Alexa Fluor 594 or donkey antimouse 488) were purchased from Molecular Probes or Jackson ImmunoResearch, respectively. Nuclei were stained with DAPI (ThermoFisher) and coverslips were mounted on glass slides. Images were generated using a Zeiss LSM 700 confocal microscope and processed using ZEN software.

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