L-Domain Flanking Sequences Are Important for Host Interactions and Efficient Budding of Vesicular Stomatitis Virus Recombinants

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Vesicular stomatitis virus (VSV) possesses a PPPY and a PSAP motif within the matrix (M) protein. The PPPY motif has significant L-domain activity in BHK-21 cells, whereas the PSAP motif does not. Since the core PSAP motif alone is insufficient to provide L-domain activity, we modified upstream or downstream amino acids flanking the PSAP core motif to determine their effect on L-domain activity. VSV recombinants were recovered that contained single or multiple amino acid mutations in upstream or downstream sequences flanking the PSAP core. Recombinant viruses were examined for growth kinetics, budding efficiency, and functional interactions with host proteins. We demonstrate that the composition of amino acids surrounding the L-domain core motifs are critical for efficient L-domain activity and for interactions with host proteins in the context of a VSV infection.

Many enveloped RNA viruses utilize late budding domains (L domains) present within their matrix (M) or M-like proteins for efficient budding from infected cells (for a review, see reference 4). Viral L domains are known to interact with specific host proteins, and these virus-host interactions allow for more efficient virus budding. Two of the best-characterized L-domain core motifs are PPPY and PT/SAP. The PPPY motif is known to interact with WW domain-containing ubiquitin ligases, such as Nedd4, and other members of the HECT family of E3 ubiquitin ligases (1, 8–10, 15, 16, 22, 24, 25, 27). The PT/SAP motif is known to interact directly with tsg101, a component of ESCRT-I and the MVB sorting pathway within mammalian cells (2, 6, 10, 14, 18–21, 26).

A number of viral M proteins possess multiple L-domain motifs (4), although not all motifs have been shown to function as L domains. Interestingly, the vesicular stomatitis virus (VSV) M protein possesses both a PPPY (amino acids 24 to 27) and a PSAP (amino acids 37 to 40) core motif. We and others have demonstrated that the PPPY motif is indeed a functional L domain; however, the downstream PSAP motif did not display L-domain activity similar to that of the PPPY motif in VSV-infected BHK-21 cells (3, 7, 11–13). Notably, conversion of the PSAP core sequence of VSV M to a PTAP core was also not sufficient to rescue budding of a PPPY mutant virus (11). These findings indicate that sequences other than the core motif are important for efficient L-domain activity. Although the importance of sequences flanking L-domain core motifs to virus budding has not been studied extensively, one recent study by Pornillos et al. examined the residues of human immunodeficiency virus type 1 (HIV-1) p6Gag that were critical for interactions with the UEV domain of host tsg101 (20). These authors demonstrated that the PTAP residues were crucial for interactions with tsg101 and

that mutations in sequences flanking the PTAP core were found to have a more modest effect on tsg101 binding and subsequent budding of HIV-1 (6, 18).

Since the presence of only the PSAP core motif in VSV M was not sufficient to rescue the budding of a PY mutant virus, we hypothesized that amino acid sequences flanking the PSAP core motif most likely were important for overall L-domain function. In this study, VSV recombinants were recovered using reverse genetics to determine whether flanking sequences are indeed critical for L-domain function. Single or multiple amino acid changes were introduced both upstream and downstream of the PSAP motif. Our results demonstrate that the amino acids surrounding the PSAP core in VSV M are crucial for determining whether a core motif possesses L-domain activity. Indeed, by changing the flanking sequences, the PSAP core motif was converted to a functional L domain that was capable of rescuing the budding defect of a PY mutant virus and capable of binding to and packaging endogenous tsg101. These results illustrate the importance of L-domain flanking residues in host interactions and in budding of VSV.

MATERIALS AND METHODS

Cells, viruses, and antibodies. BHK-21 and 293T cells were maintained in Dulbecco's minimum essential medium (DMEM; Life Technologies, Rockville, Md.) supplemented with 10% fetal bovine serum (Life Technologies) and penicillin-streptomycin (Life Technologies) at 37°C. All VSV recombinants and a recombinant vaccinia virus (VvT7) were propagated in BHK-21 cells. All virus stocks were titrated by standard plaque assay on BHK-21 cells, and all viruses contain arginine at position 51 of the M protein. Monoclonal antibody (MAb) 23H12, specific for the M protein of VSV, was kindly provided by D. S. Lyles (Wake Forest University School of Medicine, Winston-Salem, N.C.). Anti-tsg101 MAb 4A10 (Gene Tex) was used according to protocols of the suppliers.

Construction and recovery of VSV recombinants. Plasmid pVSV-FL, encoding full-length VSV cDNA (Indiana serotype), was kindly provided by J. K. Rose (Yale University School of Medicine, New Haven, Conn.). Construction of chimeric M6PY>A4 and PY>A4 genes were described previously (11), and mutations were introduced into these genes using a standard PCR technique to yield M6, PY>A4-E34R, -I41P, -MEYA>SRLE, -IDK>PEE, M6PY>A4-S33A, -S33M, -R34A, -R34E, and -SR>ME genes. These genes were inserted back into pVSV-FL to generate the full-length cDNA clones used to recover infectious virus.

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Virion protein profiles. BHK-21 cells in six-well plates were infected with VSV recombinants at a multiplicity of infection (MOI) of 10. After a 1-h incubation at 37°C, inocula were removed and cells were washed with phosphate-buffered saline three times and then incubated in serum-free DMEM at 37°C for 8 h. Culture medium was harvested and clarified at 3,000 rpm for 10 min. Virions were then centrifuged at 36,000 rpm for 2.5 h through a 20% sucrose cushion. The pellet was suspended in sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) sample buffer (125 mM Tris-HCl [pH 6.8], 4.6% SDS, 10% 2-mercaptoethanol, 0.005% bromophenol blue, 20% glycerol) and analyzed by SDS-PAGE (8%), followed by staining with GelCode Blue Stain Reagent (Pierce). Cell lysates were also prepared and analyzed by Western blotting using anti-M MAb.

One-step growth curve of VSV recombinants. BHK-21 cells in six-well plates were infected with VSV mutants at an MOI of 10. After a 1-h incubation at 37°C, inocula were removed and cells were washed with 1× phosphate-buffered saline three times and incubated with DMEM containing 10% fetal bovine serum at 37°C. At the designated time points, culture medium was harvested and titrated in duplicate by a standard plaque assay on BHK-21 cells.

Detection of endogenous host tsg101 in virions. Virion samples were prepared as described above. Virion samples were subjected to SDS-PAGE (8%), followed by Western blotting using anti-tsg101 MAb. Virion samples were also subjected to SDS-PAGE (8%), followed by staining with GelCode reagent to confirm that identical amounts of total protein for each virion sample were loaded on the gel.

siRNA transfection and VSV infection. Small interfering RNA (siRNA) transfection and VSV infection were performed as described previously (10, 12). Briefly, human 293T cells cultured in six-well plates were transfected with a combination of 0.2 µg of tsg101-specific, or nonspecific (NS) siRNA (Dharmacon Inc.) using Lipofectamine 2000 (Invitrogen). At 24 h posttransfection, cells were transfected a second time in an identical manner. After 12 h, cells were infected with VSV at an MOI of 0.01. At 6 h postinfection (p.i.), culture medium was harvested and the virus yield was determined by plaque assay on BHK-21 cells. Inhibition of tsg101 expression by siRNA was confirmed by Western blotting using anti-tsg101 MAb as described previously (10).

RESULTS

The previously characterized PY>A4 recombinant virus is known to be defective in budding. The presence of either a PSAP or a PTAP motif at amino acids 37 to 40 of VSV M in recombinant PY>A4 did not rescue the budding defect of this recombinant virus. In contrast, insertion of the PTAP core motif and flanking residues derived from p6Gag of HIV-1 into the PY>A4 recombinant at residues 33 to 44 of M protein did rescue the budding of PY>A4 to wild-type levels (11) (Fig. 1). Indeed, recombinant M6PY>A4 (11) and recombinant M6 were able to replicate and bud at levels similar to those of wild-type VSV (VSV-WT) (Fig. 1). Thus, in the context of a VSV infection, L-domain flanking sequences are clearly important for determining L-domain activity and for budding of VSV recombinants.

We next sought to determine whether single amino acid changes in the L-domain flanking sequences would be sufficient to convert the inactive PSAP core motif of VSV to an active L domain capable of rescuing the budding of the PY>A4 recombinant virus. Although single amino acid changes to flanking sequences upstream and downstream of the PTAP motif of HIV-1 Gag did not significantly affect the budding of HIV-1, VSV is unique in that it contains a negatively charged residue (E34) at position -3 upstream of the PSAP motif. To determine whether amino acid position 34 was important for L-domain activity, we mutated this residue, as well as downstream residues. Glutamic acid 34 and isoleucine 41 flanking the PSAP core motif of VSV M were changed to arginine and proline, respectively, which are present in identical positions flanking the PTAP core motif of p6Gag (Fig.

FIG. 1. Growth kinetics of the M6, M6PY>A4, and recombinant viruses, which possess single amino acid substitutions at positions 34 and 41 of the M protein of the PY>A4 virus. (A) Diagram of the VSV genome with the M gene highlighted in gray. The VSV M protein (amino acids 1 to 229) is expanded, and the sequences of amino acids 20 to 31 and amino acids 33 to 44 are indicated. The dotted line indicates that the VSV-WT M sequence is maintained. The amino acid sequence within the L domain region (amino acids 20 to 31) is shown for VSV-WT, PY>A4, M6, M6PY>A4, PY>A4-E34R, and PY>A4-I41P. (B) Graph of growth kinetics and titers of the viruses shown above at the indicated times p.i. of BHK-21 cells. Each titer represents the average of at least two independent experiments.

1A). VSV recombinants possessing these single amino acid changes were recovered in the PY>A4 background (Fig. 1A). The growth curves of these two recombinants in BHK-21 cells were virtually identical to that of PY>A4 (Fig. 1B). These results indicate that single amino acid changes at these positions were not sufficient to convert the inactive VSV PSAP motif to an active L domain.

In our next series of experiments, an approach opposite to that described above was taken. That is, rather than attempt to convert the inactive PSAP motif of VSV into an active L domain by altering flanking sequences, we attempted to inactivate the p6Gag PTAP L domain by introducing single amino acid mutations into the flanking sequences. For these studies, we focused on amino acids 33 and 34, which are upstream of the p6 L domain in VSV recombinant M6PY>A4 (Fig. 2A).

FIG. 2. Growth kinetics of M6PY>A4 recombinant viruses, which possess single or double amino acid substitution at positions 33 and 34 of the M protein of the M6PY>A4 virus. (A) The amino acid sequence within the L domain region (amino acids 20 to 31) is shown for VSV-WT, M6PY>A4, M6PY>A4-S33A, -S33M, -R34A, -R34E, and -SR>ME as shown in Fig. 1. (B) Graph of growth kinetics and titers of the viruses shown above at the indicated times p.i. of BHK-21 cells. Each titer represents the average of at least two independent experiments.

HIV-1-derived Ser-33 was changed to either Ala-33 in recombinant M6PY>A4-S33A or VSV-derived Met-33 in recombinant M6PY>A4-S33M (Fig. 2A). Similarly, HIV-1-derived Arg-34 was changed to either Ala-34 in recombinant M6PY>A4-R34A or VSV-derived Glu-34 in recombinant M6PY>A4-R34E (Fig. 2A). Lastly, VSV recombinant M6PY>A4-SR>ME was generated in which both Ser-33 and R-34 from p6Gag were changed to the VSV counterparts Met-33 and Glu-34 (Fig. 2A). Each of these recombinant viruses and the parental M6PY>A4 virus were assayed for growth kinetics in BHK-21 cells (Fig. 2B). As described above, single amino acid changes did not result in significant alterations in growth properties and budding of these recombinants in BHK-21 cells. Titers of the double mutant M6PY>A4-SR>ME were slightly reduced compared to those of the parental virus M6PY>A4 (Fig. 2B). It should be noted that none of the recombinants illustrated in Fig. 2A replicated to titers below that of the PY>A4 virus (data not shown).

FIG. 3. Growth kinetics of the recombinant viruses, which possess either the upstream four or the downstream three amino acids of the PTAP motif from HIV-1 p6Gag in place of those of the PSAP motif of VSV. (A) The amino acid sequence within the L-domain region (amino acids 20 to 31) is shown for VSV-WT, PY>A4, PY>A4-MEYA>SRLE, and PY>A4-IDK>PEE as shown in Fig. 1. (B) Graph of the growth kinetics and titers of the viruses shown above at the indicated times p.i. of BHK-21 cells. Each titer represents the average of at least two independent experiments.

Since single amino acid changes to L-domain flanking sequences had only modest effects on L-domain activity, multiple amino acids either upstream or downstream of the PSAP motif of VSV were altered to generate two additional VSV recombinants. Recombinant virus PY>A4-MEYA>SRLE contains the SRLE upstream flanking sequences of p6Gag in place of the MEYA upstream flanking sequences of VSV M, and recombinant virus PY>A4-IDK>PEE contains the PEE downstream flanking sequences of p6Gag in place of the IDK downstream sequences of VSV M (Fig. 3A). Unlike the results obtained with single amino acid mutations, modifications of multiple amino acids both upstream and downstream of the PSAP motif of VSV resulted in rescue of the budding defect of the PY>A4 virus (Fig. 3B). Indeed, the growth curves of both PY>A4-MEYA>SRLE and PY>A4-IDK>PEE recombinants were virtually identical to that of VSV-WT. Thus, modification of multiple amino acid sequences flanking the core motif directly affects L-domain activity and virus budding. Virion protein profiles were examined for VSV-WT and representative recombinant viruses (Fig. 4). As expected, the

FIG. 4. Characterization of virion protein profiles and M protein synthesis in infected BHK-21 cells. Virions released from BHK-21 cells at 8 h p.i. were purified and subjected to SDS-PAGE analysis. The G, N/P, and M proteins are indicated for VSV-WT (lane 1), PY>A4 (lane 2), M6 (lane 3), M6PY>A4 (lane 4), PY>A4-E34R (lane 5), PY>A4-I41P (lane 6), M6PY>A4-S33M (lane 7), M6PY>A4-S33A (lane 8), M6PY>A4-R34E (lane 9), M6PY>A4-R34A (lane 10), M6PY>A4-SR>ME (lane 11), PY>A4-MEYA>SRLE (lane 12), and PY>A4-IDK>PEE (lane 13). The L protein is not shown. Infected cell lysates were analyzed by Western blotting using anti-M MAb (lanes 14 to 26).

level of virion proteins was reduced for recombinants PY>A4 (lane 2), PY>A4-E34R (lane 5), and PY>A4-I41P (lane 6) that were defective in budding. In contrast, the virion protein profiles of budding competent viruses were virtually identical to that of VSV-WT (Fig. 4, lanes 1, 3, 4, and 7 to 13). As a control for protein expression, the amounts of M protein detected in all cell extracts were found to be virtually identical (Fig. 4, lanes 14 to 26).

We next sought to determine whether L-domain flanking sequences were important for packaging and interacting with host proteins involved in budding. More specifically, we sought to determine whether efficient budding of the PY>A4-MEYA>SRLE and PY>A4-IDK>PEE recombinants was due to a newly acquired ability to interact with host tsg101 and the MVB sorting machinery. Since the PT/SAP L-domain motif is known to interact physically and functionally with host tsg101, we determined whether endogenous tsg101 was packaged into budding virions and whether endogenous tsg101 was necessary for efficient budding of representative VSV recombinants (Fig. 5). BHK-21 cells were infected with VSV-WT, M6, M6PY>A4, M6PY>A4-MEYA>SRLE, or M6PY>A4-IDK>PEE, and virions were harvested and purified at 8 h p.i. The presence of endogenous tsg101 in virions was determined by Western blotting. As reported previously (10), endogenous tsg101 was virtually undetectable in VSV-WT virions (Fig. 5, lane 1); however, endogenous tsg101 was readily detected in M6, M6PY>A4, M6PY>A4-MEYA>SRLE, and M6PY>A4-IDK>PEE virions (lanes 2 to 5). Similar results were obtained using human 293T and HeLa cells (data not shown). Although the amount of tsg101 present in M6PY>A4-MEYA>SRLE and M6PY>A4-IDK>PEE (lanes 4 and 5) was slightly less than that present in M6 and M6PY>A4 virions (lanes 2 and 3), these results suggest that altering the

FIG. 5. (A) Packaging of endogenous tsg101 into virions. Western blotting using anti-tsg101 antiserum to detect endogenous tsg101 packaged into VSV-WT (lane 1), PY>A4-MEYA>SRLE (lane 2), PY>A4-IDK>PEE (lane 3), M6 (lane 4), M6PY>A4 (lane 5), and endogenous tsg101 in uninfected BHK-21 cells is also shown as a control (lane 6). Equivalent amounts of virion proteins were shown to be loaded onto the gel (data not shown). (B) tsg101 siRNA inhibits the budding of active PT/SAP motif-containing recombinants. Human 293T cells were first transfected with tsg101-specific siRNA or an NS siRNA and then infected with PS>A4, M6, M6PY>A4, PY>A4-MEYA>SRLE, or PY>A4-IDK>PEE. Virion release in the presence of NS siRNA was set at 1.0 for all viruses. Each white bar represents an average of at least three independent experiments.

flanking sequences of PSAP (MEYA>SRLE and IDK>PEE) enabled the M protein to physically interact with and package host tsg101. It is likely that this enhanced M-tsg101 interaction was sufficient to overcome the PY>A4 mutation and thus allow for budding of M6PY>A4-MEYA>SRLE and M6PY>A4-IDK>PEE to WT levels (Fig. 3).

To demonstrate further that the M-tsg101 interaction was biologically relevant for efficient budding of VSV recombinants, siRNAs were used to inhibit expression of endogenous tsg101 in virus-infected cells (Fig. 5B). A NS siRNA control and a tsg101-specific siRNA were transfected into human 293T cells, followed by infection with the indicated virus (Fig. 5B). As reported previously (10, 12), expression of endogenous tsg101 was inhibited by >90% (data not shown), and disruption of endogenous tsg101 expression by siRNA did not significantly affect the budding of VSV-WT. Similarly, inhibition of tsg101 expression only reduced titers of recombinants PS>A4, in which the PSAP motif of M protein was replaced with four alanines (11), and M6 by <2.0-fold compared to those determined in the presence of the NS siRNA (Fig. 5B). In contrast, inhibition of tsg101 expression reduced the titers of recombinants M6PY>A4, M6PY>A4-MEYA>SRLE, and M6PY>

A4-IDK>PEE by approximately fivefold (Fig. 5B). Thus, those recombinants possessing only a PT/SAP-type core motif in the proper context were capable of packaging *tsg101* into virions and were dependent on *tsg101* expression for efficient budding (Fig. 5). Interestingly, the M6 recombinant, which possesses both a PTAP (HIV-1 derived) and a PPxY (VSV derived) core motif was able to package endogenous *tsg101* but was not as dependent on *tsg101* expression for efficient budding as were the M6PY>A4, M6PY>A4-MEYA>SRLE, and M6PY>A4-IDK>PEE recombinant viruses (Fig. 5B).

DISCUSSION

Viral L domains are required for efficient budding of many RNA-containing viruses. L domains are composed of core motifs (PT/SAP, PPxY, YxxL, FIPV) that mediate interactions with host proteins to promote virus-cell separation (23; for a review, see reference 5). While the core L-domain motifs are clearly important for L-domain activity, the contributions of the residues flanking these core motifs to virus budding has largely been unexplored. The context in which the core motifs resides is likely to contribute to the overall function of the L domain. The PT/SAP-type core motif has been studied extensively, particularly that present in the p6 region of HIV-1 Gag. In an elegant study by Pornillos et al. (20), the PTAP (or PSAP) core of HIV-1 Gag was shown to physically interact with specific residues of host protein *tsg101*. While the PTAP core was critical for this virus-host interaction, sequences flanking the viral PTAP motif were thought to play a more modest role in this virus-host interaction (6, 18).

We have reported previously that the PSAP motif in VSV M does not possess L-domain activity similar to that of the PPxY motif of VSV M in BHK-21 cells (11). For example, mutation of the PSAP motif to four alanines did not significantly affect the budding of VSV (11). In addition, changing the PSAP core to a PTAP core did not rescue the budding of the PY>A4 recombinant virus (11). Lastly, our data suggest that the PSAP core motif in the M protein of VSV-WT does not interact with endogenous *tsg101* and subsequently *tsg101* is not packaged to appreciable levels in budding VSV-WT virions (10). Interestingly, insertion of a bona fide PTAP-type L domain and flanking sequences from HIV-1 p6 in place of the PSAP region of VSV M (amino acids 33 to 44) was able to rescue budding of the PY>A4 recombinant virus. Taken together, these results strongly suggest that the presence of a core L-domain motif alone is not sufficient for L-domain activity, but rather that amino acids flanking an appropriate L-domain core motif are crucial for optimal L-domain activity.

In this study, we generated a number of VSV recombinants to assess the role of amino acids flanking the L-domain core in virus budding. Single and multiple amino acids mutations were introduced both upstream and downstream of a PT/SAP core motif between amino acid positions 33 and 44 within the M protein of VSV. Overall, our results indicated that single amino acid changes within the flanking amino acids did not significantly enhance or disrupt L-domain activity. For example, changing single amino acids flanking the PSAP core of VSV to the corresponding amino acid flanking the PTAP core of HIV-1 was insufficient at converting the PSAP core of VSV to an active L domain (Fig. 1). Similarly, conversion of HIV-1

flanking amino acids in recombinant M6PY>A4 to those corresponding to VSV were unable to significantly disrupt the L-domain activity of the PTAP motif of HIV-1 in recombinant M6PY>A4 (Fig. 2). It has also been reported that single or double amino acid changes in the amino acids flanking the PTAP motif of HIV-1 p6Gag do not significantly reduce virus budding (4, 18). In contrast, multiple amino acid changes to either upstream or downstream flanking sequences did have a significant influence on L-domain activity. For example, recombinant virus M6PY>A4-MEYA>SRLE contains the parental PSAP core motif of VSV M and downstream amino acids; however, the upstream four amino acids (MEYA) were replaced with the corresponding upstream four amino acids (SRLE) normally present in p6Gag of HIV-1. This four-amino-acid change was sufficient to convert the normally inactive PSAP motif of VSV M to an active L domain. Indeed, the M6PY>A4-MEYA>SRLE recombinant was able to replicate and bud to titers that were virtually identical to those of VSV-WT in BHK-21 cells (Fig. 3). Importantly, this recombinant was now capable of interacting with and packaging endogenous *tsg101* into budding virions, and efficient budding of this recombinant was dependent on the expression of endogenous *tsg101* in infected cells (Fig. 5). Similar results were obtained with recombinant virus M6PY>A4-IDK>PEE, in which the downstream flanking sequences were modified from VSV-derived sequences to those derived from p6 of HIV-1. Thus, in the context of a VSV infection, sequences flanking the L-domain core are crucial for host interactions and efficient budding. Interestingly, recombinant virus M6, which contains a functional PPPY motif normally present in VSV and a functional PTAP motif plus flanking amino acids derived from p6 of HIV-1, was able to package *tsg101* into virions but was not as dependent on *tsg101* expression for budding as were recombinant viruses containing only a PTAP motif (Fig. 5). These findings suggest that the parental PPPY motif of VSV may be the more dominant L domain in the M6 recombinant virus. However, packaging of endogenous *tsg101* into M6 virions suggests that *tsg101* and likely other components of the MVB sorting pathway are involved in the budding of the M6 virus. A role for *tsg101* in the budding of the M6 recombinant virus may be minimal in the presence of a functional PPxY motif and may be beyond detection in this assay. Alternatively, a physical interaction with a particular host protein may not always correlate with a functional interaction. Recently, Martin-Serrano et al. reported context-dependent function of viral L domains, and function of the overlapping PTAPPEY L domains of Ebola virus M protein (VP40) is independent of *tsg101* in the context of murine leukemia virus but dependent in the context of HIV-1 (17). These results imply that *tsg101*-dependent and -independent viruses may utilize distinct cellular pathways for budding.

We are currently utilizing the M6 virus and other recombinants to elucidate further which host proteins and/or machinery are biologically relevant for budding of VSV recombinants containing various types of L-domain core motifs. It will also be of interest to determine the contributions of amino acids flanking the PPxY, YxxL, and FPIV core L-domain motifs to virus budding. In sum, these results indicate that in the context of a VSV infection, the core L-domain motifs are not sufficient to provide optimal L-domain activity. Rather, the context in

which the core motif is placed appears to be crucial for efficient budding of VSV recombinants. The identity of an optimal context and the role that these flanking amino acids play in mediating direct interactions with host proteins are under investigation.

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