Genetic Informational RNA Is Not Required for Recombinant Prion Infectivity

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Whether a genetic informational nucleic acid is required for the infectivity of transmissible spongiform encephalopathies is central to the debate about the infectious agent. Here we report that an infectious prion formed with bacterially expressed recombinant prion protein plus synthetic polyriboadenylic acid and synthetic phospholipid 1-palmitoyl-2-oleoylphosphatidylglycerol is competent to infect cultured cells and cause prion disease in wild-type mice. Our results show that genetic informational RNA is not required for recombinant prion infectivity.

The central issue in the debate about the infectious agent in transmissible spongiform encephalopathy (TSE, or prion disease) is whether the genetic information is encoded by the aberrantly folded prion protein (PrP) or a nucleic acid (6, 9, 13, 15). The prion hypothesis postulates that the infectious agent is an altered conformer of PrP (PrP^{Sc}), which seeds the conversion of normal host-encoded PrP (PrP^C) into the PrP^{Sc} isoform and causes prion disease (15). In contrast, the viral hypothesis posits that the properties of the agent are specified by nucleic acids (6, 9, 13). Although the involvement of a large viral genome in prion infectivity was ruled out decades ago (1), the virino model argues that the infectious agent could be a nucleic acid-PrP complex (6, 9), in which a small noncoding nucleic acid dictates the disease phenotype and the disease-associated aggregated PrP^{Sc} protects the genetic informational nucleic acid. The presence of small RNAs in infectious particles purified from diseased brains (16) and the expanding list of biologically functional noncoding small RNAs (3, 8) foster the speculation that the genetic information of the TSE agent might be carried by small RNAs (genetic informational RNAs). However, the notion that a genetic informational RNA is required for prion infectivity is contradictory to several recent studies (5, 7, 10, 12, 14, 18).

Using protein misfolding cyclic amplification (PMCA) (2), we previously showed that, in the presence of synthetic phospholipid 1-palmitoyl-2-oleoylphosphatidylglycerol (POPG) and total RNA isolated from normal mouse liver, bacterially expressed recombinant prion protein (rPrP) was converted into an altered conformational state that caused prion disease in wild-type mice, with an incubation period comparable to that observed with naturally occurring prions (18). Because some rPrP conformers acquired proteinase K (PK) resistance after PMCA (rPrP-res), we have used rPrP-res to represent altered rPrP conformational states in PMCA products, which does not necessarily imply that the infectious prion conformation has to be PK resistant.

The use of tissue-derived RNA leaves open the possibility that, as proposed by the virino model (6, 9), a conjectured informational RNA present in host tissue might specifically bind to the converted rPrP-res form, which would serve as its protective coat. To address this possibility, we modified our PMCA protocol (17, 18) to allow a more robust propagation of rPrP-res with polyriboadenylic acid [poly(rA)] and POPG (Fig. 1A) (see the supplemental material for a description of our methods). We eliminated

RNA molecules in the original total RNA-generated seed (18) by propagating rPrP-res with poly(rA) and POPG for more than 20 rounds, which resulted in the original seed being diluted >10²⁰-fold. The rPrP-res thus propagated with poly(rA) and POPG, named rPrP-res^{poly(rA)}, was aggregated and had a highly PK-resistant C terminus (Fig. 1B and C). To minimize cross-contamination, rPrP-res^{poly(rA)} was propagated in a designated PMCA machine.

The rPrP-res^{poly(rA)} was able to infect mouse neuronal CAD5 cells (11) and converted endogenous glycosylated PrP^C into an aggregated and PK-resistant conformer (Fig. 1D). Wild-type CD-1 mice inoculated intracerebrally (i.c.) with rPrP-res^{poly(rA)} developed symptoms of murine prion disease around 190 days postinoculation (dpi), including clasping, bradykinesia, tail plasticity, kyphosis, and mild ataxia, and reached the terminal stage of the disease at 209 to 244 dpi (Fig. 2A). Two independent rPrP-res^{poly(rA)} inoculations resulted in similar survival times, which were 220 ± 4.5 days (mean ± standard error of the mean) and 228 ± 4.5 days (see the table in the supplemental material). Biochemical and histopathological analyses revealed the presence of PK-resistant PrP^{Sc}, spongiosis, gliosis, and the deposition of abnormal PrP in the brain (Fig. 2B and C), confirming that these mice were suffering from prion disease.

Control CD-1 mice inoculated i.c. with PMCA substrate composed of rPrP, POPG, and poly(rA) that had not been subjected to the PMCA reaction remained healthy for more than 325 days (Fig. 2A). Brains of these control mice were devoid of PrP^{Sc} (Fig. 2B) and did not show spongiosis, gliosis, or abnormal PrP deposition (Fig. 2C). The other control group, CD-1 mice inoculated i.c. with rPrP-res^{RNA} (rPrP-res propagated with total mouse liver RNA), developed symptoms around 150 dpi and, on average, survived 164 ± 5.7 days.

Received 6 September 2011 Accepted 8 November 2011

Published ahead of print 16 November 2011

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Supplemental material for this article may be found at <http://jvi.asm.org/>.

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doi:10.1128/JVI.06216-11

FIG 1 Formation of rPrP-res^{Poly(rA)}. (A) A representative result of serial PMCA propagation of rPrP-res^{Poly(rA)} with rPrP, POPG, and poly(rA). PMCA products were digested with 50 μg PK/ml. C, undigested rPrP. (B) The rPrP-res^{Poly(rA)} PMCA products were digested with the indicated concentrations of PK, and the molar ratios for rPrP:PK were 1:1.6, 1:3.2, 1:6.4, and 1:10. (C) The rPrP-res^{Poly(rA)} PMCA products were subjected to 100,000 × g centrifugation at 4°C for 1 h. The rPrP in the supernatant (S), pellet (P), and total input (T) and the PK-resistant rPrP in supernatant and pellet were detected by immunoblot analysis with POM1 antibody (left panel) or 8B4 antibody (right panel) as indicated. All PK digestions were carried out at 37°C for 30 min. (D) CAD5 cells were infected with rPrP-res^{Poly(rA)} or mock infected with Opti-MEM only. Cells at the indicated passages were lysed, digested with 25 μg PK/ml at 37°C for 60 min, and centrifuged at 100,000 × g for 1 h. The pellet was analyzed by SDS-PAGE. C1, undigested CAD5 cell lysate; C2, pellet of PK-digested, uninfected CAD5 cell lysate. PrP was detected by immunoblot analysis with POM1 anti-PrP antibody.

Interestingly, the survival times for rPrP-res^{Poly(rA)}-inoculated mice were significantly longer than those for rPrP-res^{RNA}-inoculated mice. It is unlikely that variability in the inoculation caused the extension of incubation time, because the survival

times for two independent rPrP-res^{Poly(rA)} inoculations were almost identical (Fig. 2A). The extended incubation time may have come about because the modified PMCA protocol is not optimal for converting rPrP into the infectious conformation and/or

FIG 2 Mouse bioassay. (A) Survival plot of CD-1 mice inoculated with rPrP-res^{Poly(rA)}, rPrP-res^{RNA}, or the control inoculum [PMCA substrate composed of rPrP, POPG, and poly(rA) but not subjected to PMCA]. One mouse injected with control inoculum was sacrificed at 241 dpi and used as a control for biochemical and histopathological analyses. (B) Brain homogenates from mice injected with the control inoculum or rPrP-res^{Poly(rA)} were subjected to PK digestion, and PK-resistant PrP was detected by immunoblot analysis with M20 anti-PrP antibody (Santa Cruz Biotechnology). C1, undigested brain homogenate; C2, PK-digested brain homogenate prepared from a mouse that received control inoculum. (C) Histopathological analyses of mice inoculated with control or rPrP-res^{Poly(rA)} inoculum. Brain sections were stained with hematoxylin and eosin (HE) to show spongiosis, SAF84 anti-PrP antibody (PrP; Cayman Chemical) to show aberrant PrP accumulation, an anti-glial fibrillary acidic protein antibody (GFAP; Dako) to show astrogliosis, or an anti-ionized calcium-binding adaptor molecule 1 antibody (Iba1; Wako Chemical USA) to show microgliosis. Bar, 100 μm.

poly(rA) might be a less efficient cofactor in our assay. Both scenarios would result in lower infectivity, which is consistent with our observation that rPrP-res^{poly(rA)} was less efficient than rPrP-res^{RNA} in infecting CAD5 cells (Fig. 1D; see also Fig. S1 in the supplemental material) and that the second-round passage of rPrP-res^{poly(rA)}-caused disease in CD-1 mice shortened the survival time to 173 ± 2.8 days (average of two experimental groups [see the table in the supplemental material]). Alternatively, since poly(rA), in contrast to natural RNA, does not form higher-order structures, it may induce and/or stabilize a PrP conformation that is different from that of rPrP-res^{RNA} and entails a longer incubation time. Notably, brains of rPrP-res^{poly(rA)}-inoculated mice exhibited a milder extent of spongiosis and weaker SAF84 antibody staining of abnormal PrP deposition compared to rPrP-res^{RNA}-inoculated mice (data not shown), but these differences need to be verified in an inbred mouse line.

Deleault et al. showed that infectious prions can be formed with purified PrP^C plus copurified lipids and synthetic poly(rA) or poly(dT) oligonucleotides (5, 14), which argues against a role of genetic informational RNA in prion infectivity. Formation of infectious prions with rPrP is widely accepted as the strongest evidence against the viral or virino hypothesis. This is because rPrP is expressed in bacteria that lack mammalian informational nucleic acids and because the key step in rPrP purification, nickel affinity chromatography in the presence 6 M guanidinium hydrochloride (17, 18), greatly minimizes the possibility of bacterial nucleic acid contamination. Amyloid fibers formed with rPrP cause prion disease in transgenic mice overexpressing PrP but not in wild-type mice (4, 10), and they induced infectious prion formation in a subset of asymptomatic wild-type hamsters (12). Recently, Kim et al. propagated partially purified PrP^{Sc} with rPrP in the absence of any mammalian cofactor and showed that the PMCA products were sufficient to cause prion disease in wild-type hamsters (7). Despite a relatively large variability in incubation times and attack rates (7), their study did meet the gold standard of rPrP-derived prions causing disease in wild-type animals.

Our results show that recombinant prions formed *in vitro* with bacterially expressed rPrP plus synthetic POPG and poly(rA) are able to cause prion disease in wild-type CD-1 mice with a relatively well-synchronized incubation time. Moreover, our finding that rPrP-res^{poly(rA)} is sufficient to infect cultured CAD5 cells eliminates the possibility that a tissue-derived component is necessary to render synthetic prions infectious. Since poly(rA) itself does not encode any meaningful genetic information, our results demon-

strate that genetic informational RNA is not required for prion infectivity.

ACKNOWLEDGMENTS

We thank Martin Jeffrey for confirming the histological findings and Adriano Aguzzi and Man-Sun Sy for providing the POM1 and 8B4 antibodies.

This work was supported by NIH grants 1R01NS060729 to J.M. and 1R01NS059543 and 1R01NS067214 and a donation from the Alafi Family Foundation to C.W. Work at Ohio State University was also partly supported by NINDS Neuroscience Core Grant P30NS045758.

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