A Determinant for Central Nervous System Persistence Localized in the Capsid of Theiler’s Murine Encephalomyelitis Virus by Using Recombinant Viruses

CECILIA ADAMI, ARTHUR E. PRITCHARD, TODD KNAUF, MING LUO, and HOWARD L. LIPTON

Department of Biochemistry, Molecular Biology and Cell Biology, Northwestern University, and Division of Neurology, Evanston Hospital, Evanston, Illinois; Center for Macromolecular Crystallography, University of Alabama at Birmingham, Birmingham, Alabama 35294-0005; and Department of Biochemistry, University of Colorado Health Sciences Center, Denver, Colorado 80262

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Recombinant Theiler’s murine encephalomyelitis viruses (TMEV) constructed by exchanging corresponding genomic regions between the virulent GDVII and less virulent BeAn virus cDNAs have been used to map a determinant for virus persistence to the leader P1 sequences encoding the leader and capsid proteins (2, 4, 12, 18). However, reports regarding whether this determinant can be contributed only by the less virulent TMEV or whether highly virulent strains also contribute are conflicting (4, 18). Direct assessment of GDVII virus persistence is difficult because infected animals do not survive the acute period, even at low inoculation doses, e.g., 1 50% lethal dose (LD50) (8). Use of recombinant viruses for finer-scale mapping of pathogenetic determinants within the TMEV capsid where there is extremely tight packing of amino acids in protomers is needed but may be problematic, especially considering the large number of amino acids that differ between the parents. The recombinant nature of such constructs tends to result in nonviable or growth-compromised viruses (16, 19). We now provide further evidence supporting the localization of a persistence determinant to the leader P1 (capsid) sequences. Further, recombinant viruses in which BeAn sequences progressively replaced those of GDVII within the capsid starting at the leader NH2 terminus suggest that a conformational determinant requiring homologous sequences in both the VP2 puff and VP1 loop regions, which are in close contact on the virion surface, might underlie persistence.

The demyelinating process in Theiler’s murine encephalomyelitis virus (TMEV) infection in mice requires virus persistence in the central nervous system. Using recombinant TMEV assembled between the virulent GDVII and less virulent BeAn virus cDNAs, we now provide additional evidence supporting the localization of a persistence determinant to the leader P1 (capsid) sequences. Further, recombinant viruses in which BeAn sequences progressively replaced those of GDVII within the capsid starting at the leader NH2 terminus suggest that a conformational determinant requiring homologous sequences in both the VP2 puff and VP1 loop regions, which are in close contact on the virion surface, might underlie persistence.

In vitro growth characteristics of recombinant viruses. The progeny derived from the six recombinant constructs yielding viable viruses were assessed for plaque size, virus RNA replication, and growth kinetics. Recombinant 7B produced large plaques (≈3-mm diameter), whereas 3B produced small plaques (<1-mm diameter). The four recombinants in which BeAn leader P1 (capsid) sequences progressively replaced those of GDVII on a GDVII background all produced smaller plaques, e.g., similar to those of BeAn virus. Recombinants 38...
and 40 produced plaques that were slightly larger and much smaller, respectively, than those of the other two recombinants. Viral RNA replication (not shown) and one-step virus growth kinetics for recombinants 7B and 3B more closely paralleled those of the parent that contributed the nonstructural genes, e.g., the 3D RNA polymerase (Fig. 2A). Single-step growth kinetics for recombinants 38 and 41 were similar to that of BeAn, with final virus yields of 70 to 90 PFU/cell, whereas recombinants 39 and 41 were growth delayed, with final yields of 10 PFU/cell (Fig. 2B). This suggests that recombinants 39 and 40 are defective in RNA replication or virion assembly, as demonstrated for other GDVII-BeAn capsid recombinants (16).

The minimal BeAn determinant for CNS persistence requires replacement of the entire capsid, excluding the carboxyl half of VP1 (1D). Additional SJL mice were inoculated i.c. with parental BeAn, and the recombinant viruses and monitored to days 60 to 90 postinfection (p.i.) (Table 2; Fig. 3). Of 14 mice inoculated with recombinant 3B, 13 (92%) developed signs of chronic demyelinating disease, e.g., a waddling, spastic gait, tremors, and neurogenic bladders, and 3 of 3 mice examined had extensive inflammatory demyelinating lesions throughout the spinal cord. Infectious virus was detected in the CNS in 9 of 10 mice inoculated with 3B. None of the mice inoculated with recombinants 38 or 39 developed demyelinating disease or virus persistence and demyelinating lesions; however, rare mononuclear inflammatory cell infiltrates were seen in the spinal cord white matter. While 1 of 19 mice inoculated with the recombinant 40 developed demyelinating disease, none of 8 mice had evidence of virus persistence and only 1 of 10 had demyelinating lesions. The one clinically affected recombinant 40-inoculated mouse had extensive demyelinating lesions; spinal cord tissue from this paraformaldehyde-perfused animal was not available for virus assay. Considering the low acute CNS virus titers in recombinant 40-inoculated mice, it remains unclear why this single animal developed CNS persistence and demyelination.

In contrast, 11 of 15 (70%) recombinant 41-inoculated mice developed demyelinating disease, 4 of 4 mice examined had spinal cord demyelination, and 10 of 10 developed CNS persistence (Table 2; Fig. 3). The inflammation and demyelination in recombinant 41-inoculated mice were indistinguishable

| TABLE 1. Acute virus titers in brains of mice inoculated i.c. with parental or recombinant TMEV |
|-----------------------------------------------|-----------------------------------------------|
| Expt 1 | Expt 2 |
| Parental or recombinant virus | Titera |
| GDVII | 4.5 × 10³ (2) |
| BeAn | 9.3 × 10³ (3) |
| 7B | 2.3 × 10³ (2) |
| 3B | 1.9 × 10³ (3) |
| 38 | 7.6 × 10³ (4) |
| 39 | 4.7 × 10³ (2) |
| 40 | 5.0 × 10³ (3) |
| 41 | 1.7 × 10³ (3) |

a Mice were inoculated with 10⁶ PFU of virus in the right cerebral hemisphere. Values are mean virus titers per gram of brain in mice killed on day 6 p.i. Numbers of mice are in parentheses.
from those observed for parental BeAn virus-inoculated mice. Therefore, the minimal BeAn determinant for CNS persistence appears to require almost the entire replacement of the capsid, excluding the carboxyl half of VP1.

Finer mapping of the TMEV persistence phenotype by assembling recombinants in which GDVII was progressively replaced with BeAn starting in the leader showed that persistence was restored only when BeAn extended from the leader to approximately halfway through VP1 (169 of 276 VP1 residues replaced in recombinant 41). When BeAn sequences extended only into the NH2 terminus (30 of 276 VP1 residues replaced in recombinant 39) or one-fourth of the way through VP1 (65 of 276 VP1 residues replaced in recombinant 40), virus persistence was not observed. There are at least two interpretations of this result. First, one or more of the nine BeAn VP1 residues that are different in GDVII between the MunI and MluI restriction endonuclease sites might be required for virus persistence. Although the use of recombinants between virulent and attenuated parental viruses has served to map major pathogenic determinants in other picornaviruses to a single structural amino acid (1a, 3, 15, 17), there are relatively greater amino acid differences between GDVII and BeAn in the capsid (a total of 40 residue differences [14]), arguing against the possibility that a single residue underlies TMEV persistence. Nonetheless, we are currently testing this hypothesis by mutating these GDVII residues to their BeAn counterparts in the GDVII parent. Recombinants 29, 37, and 33, which progressively replaced the GDVII capsid with BeAn starting at the 1D/2A cleavage site and intended to resolve this issue, were either neurovirulent or nonviable. Similar GDVII-DA constructs replacing GDVII with DA sequences in VP1 have also been nonviable (1). A second possibility is that since BeAn sequences in the nonpersisting recombinants 39 and 40 ended upstream of the VP1 loops, whereas in the persisting recombinant 41 BeAn nucleotides extended through the VP1 loops, BeAn persistence depends on a conformational determinant that requires homologous sequences in the VP2 puff and VP1 loops, which closely interact on the virion surface (5, 9, 10).

The idea of a conformational determinant that may involve these surface loops is supported by recent observations on the persistence of another TMEV recombinant, GD1B-2C/DAFL3. This GDVII-DA recombinant virus, which is partially neurovirulent, persists in the CNS and produces demyelination (4, 18). GD1B-2C/DAFL3 contains GDVII sequences in the carboxyl half of VP2 (amino acids 152 to 267) and in VP3 and VP1 on a DA virus background and was constructed by using the NcoI site between the sequences encoding VP2 puff A and puff B. As a result, GD1B-2C/DAFL3 has a hybrid VP2 puff, with puff A containing DA sequences and puff B containing GDVII sequences. Recently, the GDVII NcoI-AatII or 1B-2C fragment was assembled into parental DA cDNAs constructed in different laboratories (11, 13). It was shown that VP2 residue 141 on the tip of puff A was a Lys in one construct and an Asn in the other (7). GD1B-2C/DAFL3 persisted only when DA VP2 141 was a Lys (7), highlighting the location of this residue within the VP2 puff. The fact that progeny derived from the DA parental clones persisted with either Lys or Asn in this position suggests that mutations in residue 141 in the recombinant affect the conformation of downstream GDVII sequences. Most likely, because of its proximity to VP2 puff B, mutation of residue 141 changes the conformation of VP2 puff B and, indirectly, that of the VP1 loops.

Our recombinant 38, in which BeAn sequences in the capsid were replaced by GDVII in the carboxyl terminus of VP2 (amino acids 237 to 267) and in VP3 and VP1, is similar to GD1B-2C/DAFL3, except that the NH2-terminal 24 amino acids of the leader protein are from GDVII and that the VP2 puff and downstream VP2 nucleotides are entirely BeAn. Recombinant 38 is also partially neurovirulent, but surviving mice do not develop persistent CNS infections. The reason for the difference in virus persistence between GD1B-2C/DAFL3 and recombinant 38 is not readily evident, since the 24 NH2-terminal, less-virulent residues in the leader protein are not required for persistence of GDVII-BeAn recombinant 3B (this study) or GDVII-DA recombinant R2 (12). However, changes in the region of the VP2 puff may affect the conformation of its other parts or that of neighboring structures, particularly VP1 loop 2, which interacts with VP2 puff B (5, 9, 10). For example, in BeAn, the carboxyl group of VP2 puff B Asp 170 forms a hydrogen bond with the side chain of Arg 172 and the side chain of VP1 loop 2 Trp 95 (9). The latter is probably mediated by a water molecule. Similar VP2 puff B and VP1 loop 2 interactions are found in the GDVII and DA structures (5, 10). These surface loop interactions might well be altered in the recombinant viruses, yet general structural changes due to the
recombinant nature of the constructs cannot be predicted from the parental structures. Such general structural changes may also affect a putative conformational determinant. However, perhaps the most persuasive argument for the involvement of a conformational determinant of the VP2 puff and VP1 loops in persistence is the fact that both GD1B-2C/DAFL3, which contains mostly GDVII capsid sequences, and recombinant 41, which contains mostly BeAn capsid sequences, cause CNS persistence.

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