Epitope-Tagged L* Protein of Theiler’s Murine Encephalomyelitis Virus Is Expressed in the Central Nervous System in the Acute Phase of Infection

Kunihiko Asakura, Harunobu Murayama, Toshiki Himeda, and Yoshiro Ohara

Department of Microbiology, Kanazawa Medical University, Ishikawa 920-0293, and Division of Pathology, Sendai City Hospital, Sendai 984-0075

Received 19 June 2002/Accepted 9 September 2002

TO subgroup strains of Theiler’s murine encephalomyelitis virus (TMEV) synthesize L* protein from an alternative initiation codon. We first demonstrated L* expression in the central nervous system (CNS) of TMEV-infected mice during the acute phase of infection by immunoprecipitation and immunoblotting with anti-L* antibody. In addition, we generated mutant viruses which synthesize 3xFLAG epitope-tagged L* protein. With a mutant virus expressing 3xFLAG epitope-tagged L*, designated DA/3xFLAGL*, we investigated L* in the CNS in the acute phase of infection. DA/3xFLAGL* did not change the virus tropism in comparison with wild-type virus, and L* was clearly identified in the CNS in both susceptible and resistant strains of mice. Double immunolabeling studies showed that L* is colocalized with TMEV polyprotein and exclusively expressed in neurons.

Theiler’s murine encephalomyelitis virus (TMEV) is a picornavirus belonging to the Cardiovirus genus (10). TMEV is classified into two subgroups (TO and GDVII) based on differences in their biological activities. The DA strain and other members of the TO subgroup cause a biphasic disease characterized by acute self-limiting gray matter inflammation followed by chronic white matter involvement, i.e., inflammatory demyelination in the spinal cord in susceptible strains of mice (H-2d or H-2k haplotype) but not in resistant strains (H-2b haplotype) when inoculated intracerebrally (5, 6, 13). The second phase serves as an experimental model for multiple sclerosis, a human demyelinating disease of the central nervous system (CNS). In contrast to the TO subgroup, the GDVII subgroup is more neurovirulent and causes acute fatal encephalomyelitis with no demyelination (6). The precise mechanism of persistent infection and demyelination by the TO subgroup is yet to be elucidated.

Picornaviruses generally synthesize a long polyprotein with one open reading frame. However, the DA strain and other members of the TO subgroup translate another 17-kDa protein, designated L*, which is out of frame with the virus polyprotein and is initiated 13 nucleotides downstream from the AUG used to initiate the polyprotein (2, 7). The GDVII subgroup, which does not demyelinate or persist, has an ACG alternative initiation codon. We first demonstrated L* expression in the CNS of TMEV-infected mice during the acute phase of infection by immunoprecipitation and immunoblotting with anti-L* Ab. With a mutant virus expressing 3xFLAG epitope-tagged L*, designated DA/3xFLAGL*, we investigated L* in the CNS in the acute phase of infection. DA/3xFLAGL* did not change the virus tropism in comparison with wild-type virus, and L* was clearly identified in the CNS in both susceptible and resistant strains of mice. Double immunolabeling studies showed that L* is colocalized with TMEV polyprotein and exclusively expressed in neurons.

We have previously shown that L* is required for virus growth in macrophages and/or microglial cells (8, 16) in which DA is considered to persist. We also generated a polyclonal rabbit anti-L* antibody (Ab) and characterized L* in vitro. L* was not incorporated into virions (9). Immunocytochemical and immunoblotting studies with microtubules isolated from DA-infected cells have suggested that L* is associated with microtubules (9).

In this study, we focused on the acute phase of infection by DA and investigated the in vivo expression of L* in the CNS. First, we tried to confirm L* expression in the CNS by immunoprecipitation and immunoblotting directly with anti-L* Ab. The animal experiments were approved by the Institutional Animal Care and Use Committee. Female 4-week-old SJL/J mice (Jackson Laboratories, Bar Harbor, Maine) were injected intracerebrally with 2 × 10^5 PFU of DA in a 10-μl volume and were sacrificed at 3 days postinfection (p.i.). Multiple 10-μm-thick deparaffinized brain tissue sections were used for protein extraction. The protein extracted from the brain sections of uninfected SJL/J mice was used as a negative control, and the protein extracted from BHK-21 cells infected with DA was used as a positive control. L* was immunoprecipitated from the extracted protein with a Seize X protein G immunoprecipitation kit (Pierce, Rockford, Ill.) according to the manufacturer’s instructions. Briefly, affinity-purified anti-L* Ab was bound to the protein G and was immobilized by a cross-linker agent, disuccinimidyl suberate, to avoid contamination of the purified antigen with the precipitating primary Ab. The extracted protein, diluted with the provided binding buffer in the kit, was incubated with the immobilized anti-L* Ab to form the immune complex. The bound antigen (L*) was eluted by elution buffer and loaded onto a 15% polyacrylamide gel. Immunoblotting with anti-L* Ab was performed. Bound Ab was detected with biotinylated secondary antibody and alkaline...
phosphatase-conjugated streptavidin (all from Jackson Immunoresearch, West Grove, Pa.), using 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT). L* was successfully immunoprecipitated with anti-L* Ab and identified as a 17-kDa single band by immunoblotting with tissues from DA-infected SJL/J mice but not with those from uninfected control mice (Fig. 1).

Polyclonal rabbit anti-L* Ab was generated against a synthetic linear peptide of L* at amino acid residues 70 to 88 (8). Anti-L* Ab worked well against linearized protein with detergent in immunoblotting but not in immunohistochemical studies. The sections stained with affinity-purified anti-L* Ab showed faint L* staining and high background even with the pretreatment of tissue sections by trypsin, microwave, or autoclave. One possible reason for the difficulties in immunohistochemistry was that the amount of L* is extremely low in vivo and is below the level of its detection. Alternatively, L* is a hydrophobic protein, and anti-L* Ab epitope, amino acid residues 70 to 88, may be hidden inside of the native L* in the infected cells. In vitro studies with BHK-21 cells showed the synthesis of L* was far less than that of viral capsid proteins (9). However, L* was successfully identified in vivo by immunoprecipitation and immunoblotting with anti-L* Ab. Therefore, at least, the amount of L* in the CNS is enough to be detected.

To improve the detection of L* for immunohistochemical study, mutant viruses expressing FLAG epitope-tagged L* were generated. The constructs containing FLAG or 3xFLAG epitope-tag sequence in the 5' site of L*, right after the L* initiation codon which is in the leader peptide of virus polyprotein reading frame, were generated from the parental infec-

FIG. 1. Immunoprecipitation and immunoblotting with anti-L* Ab. Multiple 10-μm-thick deparaffinized brain tissue sections from a DA-infected SJL/J mouse (3 days p.i.) were used for protein extraction. The protein extracted from the brain sections of the uninfected SJL/J mouse was used as a negative control, and the protein extracted from BHK-21 cells infected with DA was used as a positive control. L* was immunoprecipitated from the extracted protein by affinity-purified L* Ab bound to the protein G and immobilized by cross-linker. Immunoprecipitated proteins were separated on an SDS-polyacrylamide gel. The bound Ig (anti-L* Ab) was detected with biotinylated secondary Abs and alkaline phosphatase-conjugated streptavidin using BCIP/NBT. Lanes: 1, SJL/J mouse infected with DA; 2, uninfected SJL/J mouse; 3, BHK-21 cells infected with DA. The arrow indicates the immunoprecipitated L*.

FIG. 2. Schematic diagram of DA/FLAGL* and DA/3xFLAGL* mutant constructs. By first-round PCR, two cDNA fragments containing FLAG or 3xFLAG sequence were amplified. By second-round PCR, two cDNA fragments were combined into one cDNA fragment carrying FLAG or 3xFLAG sequence. Amino acids are represented by single-letter code. Asterisks indicate nucleotides from original sequences that were modified so as not to have any termination codon when virus polyprotein reading frame sequence was used. Details are described in Materials and Methods.
tious cDNA clone pDAFL3 (14). For this modification, modi-

cified overlap extension by PCR was applied (18). As shown in

Fig. 2 and Table 1 by first-round PCR, two independent cDNA

fragments containing FLAG or 3xFLAG sequence were am-

plified from pDAFL3, with two sets of primers. Inserted FLAG

and 3xFLAG sequences were slightly modi-

ed from the orig-

inal FLAG and 3xFLAG sequences (pCMV-Tag vector, Strat-

gene, La Jolla, Calif., and p3xFLAG-CMV-10 vector, Sigma,

St. Louis, Mo., respectively) so as not to have any termination
codon when virus polyprotein reading frame was used. For

second-round PCR, the equimolar mixture of these purified

PCR products was amplified with a primer set. Then, the PCR

products generated by overlap extension were digested with

Eco72I (Fermentas, Hanover, Md.) and MscI (New England

Biolabs, Beverly, Mass.) and ligated to pDAFL3 digested with

Eco72I (at nucleotide [nt] 808) and MscI (at nt 1708). The

generated mutant viruses carrying FLAG or 3xFLAG-tag se-\n
quence were designated DA/FLAGL* and DA/3xFLAGL*,

respectively. The growth kinetics of mutant viruses in BHK-21

cells were examined. Wild-type DA and mutant viruses showed

similar growth kinetics. In all virus infections, the titer reached

a peak at 12 h p.i. and then gradually decreased (Fig. 3). The

peak titer of wild-type DA was 1.5- to 2-fold higher than that

of mutant viruses.

To examine the enhancement of L* detection with anti-

FLAG monoclonal Ab (MAb) in vitro, extracted proteins from

BHK-21 cells infected with DA, DA/FLAGL*, or DA/

3xFLAGL* were separated by sodium dodecyl sulfate (SDS)-
polyacrylamide gel electrophoresis under reducing conditions

on 15% acrylamide gels and immunoblotting was performed by

using alkaline phosphatase-conjugated streptavidin. L* was de-

tectable by immunoblotting with anti-L* Ab in BHK-21 cells

infected with all the mutants examined (Fig. 4A). Proteins

(equal amounts) extracted from BHK-21 cells infected with
each virus were loaded on a polyacrylamide gel. There was no

big difference in the level of L* production (Fig. 4A, lanes 1 to

3). Immunoblotting with anti-VP1 capsid protein MAb (kindly

provided from R. Roos, University of Chicago) also showed a

similar level of VP1 capsid protein production for each mutant

in virus-infected BHK-21 cells, according to the intensity of

bands (Fig. 4C). DA/FLAGL*, which has a FLAG insert in the

5’/H11032

site of L* did not change the sensitivity of L* detection upon

immunoblotting with anti-FLAG M2 MAb (Fig. 4B, lane 2). In

contrast, 3xFLAG insertion (DA/3xFLAGL*) drastically en-
hanced the detection of L* and showed a dense band of L*

upon immunoblotting with anti-FLAG M2 MAb (Fig. 4B, lane

3). Therefore, for further study, DA/3xFLAGL* was used for

L* detection in the CNS. The identification of FLAG-L* and

3xFLAG-L* as duplet bands by immunoblotting both with

anti-L* and anti-FLAG Abs may be due to posttranslational

modification.

Female SJL/J mice (susceptible strain; Jackson Laborato-

TABLE 1. Primers used for overlap extension PCR

| Primer | Sequence
|---|---
| **For FLAG sequence insertion** |  |
| **First-set primers** |  |
| Forward | 5’-AACATGCAGAGTAACGCGAAG-3’ (nt 676–696) |
| Reverse | 5’-GTATCCTTGCTGCTTGCTTCTTGAATCCATGTTTGCAAGCCATAGTGT-3’ |
| **Second-set primers** |  |
| Forward | 5’-ACATGGATTACAAAGGAGCGACGACGAGAAAGAGAAG-3’ (nt 671–700) |
| Reverse | 5’-ATCCCCGTGTTGCTTTTGAGTCTCATGTTTTGCAAGCCATAGTGCAAT-3’ |
| **Second-round PCR** |  |
| Forward | 5’-ACATGGACTACAAAGACCAGCGGAGATTACAAAGTACACATGCGTATTACAGGAGCAAGCAGACCGAAUGGATCTCAGATGTGAGCTGCCC-3’ |
| Reverse | 5’-AGACACCTCCGTCCTCAGCCAG-3’ (nt 1792–1772) |
| **For 3xFLAG sequence insertion** |  |
| **First-set primers** |  |
| Forward | 5’-CGTGCAACATGCAGAGTAACGCGAAGAAAG-3’ (nt 671–700) |
| Reverse | 5’-ATCCCCGTGTTGCTTTTGAGTCTCATGTTTTGCAAGCCATAGTGCAAT-3’ |
| **Second-set primers** |  |
| Forward | 5’-ACATGGACTACAAAGACCAGCGGAGATTACAAAGTACACATGCGTATTACAGGAGCAAGCAGACCGAAUGGATCTCAGATGTGAGCTGCCC-3’ |
| Reverse | 5’-AGACACCTCCGTCCTCAGCCAG-3’ (nt 1792–1772) |
| **Second-round PCR** |  |
| Forward | 5’-AACATGCAGAGTAACGCGAAG-3’ (nt 676–696) |
| Reverse | 5’-AGACACCTCCGTCCTCAGCCAG-3’ (nt 1792–1772) |

*FLAG and 3xFLAG sequences are underlined.

FIG. 3. Growth kinetics of wild-type DA and mutants on BHK-21 cells. The culture supernatants and cell lysates of infected cells at various time points were subjected to titer determination by a plaque assay. Values represent the mean ± standard deviation of triplicate samples.
ries) and female C57BL/6 mice (resistant strain; Charles River Japan, Yokohama, Japan) from 4 weeks of age were inoculated intracerebrally with 2 x 10^6 PFU of wild-type DA or DA/3xFLAGL* mutant virus in a volume of 10 μl. At 3, 5, and 7 days p.i., mice were sacrificed and perfused with saline followed by treatment with 10% formalin. The formalin-fixed brain tissues were dehydrated and embedded in paraffin. Five coronal sections of the CNS were studied for immunohistoch- emistry, including sections of the cerebrum, cerebellum, and brain stem of each mouse inoculated with DA/3xFLAGL* or wild-type DA virus. Four-micrometer-thick paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated through ethanol to water. Tissue sections were pretreated in an autoclave for 10 min at 121°C. The following Abs were used: mouse anti-FLAG M2 MAb for FLAG-tagged L*, rabbit polyclonal anti-TMEV Ab (kindly provided by M. Njenga, University of Minnesota), mouse anti-VP1 capsid protein MAb, rabbit polyclonal anti-glial fibrillary acidic protein (GFAP) Ab (DAKO, Carpinteria, Calif.) for astrocytes, rabbit polyclonal anti-human glutathione S-transferase (GST)-π Ab (MBL, Nagoya, Japan) for oligodendrocytes (17), and mouse antineuronal nuclei (NeuN) MAb (Chemicon, Temecula, Calif.) for neurons. To identify macrophages/microglial cells, biotinylated BS-1 lectin (Vector Laboratories, Burlingame, Calif.) was used. All sections were counterstained with hematoxylin.

First, single immunolabeling with anti-TMEV Ab or antiFLAG MAb was performed to examine the virus distribution and L* expression. In both SJL/J and C57BL/6 mice, the viral antigen and L* were not detected in brain stem and cerebellum at any time point (3, 5, and 7 days p.i.). There was no difference between DA/3xFLAGL* virus and wild-type DA virus in the anatomical distribution of viral antigens and inflammatory lesions in the CNS. At 3 days p.i., viral antigen and L* were identified mostly in the gray matter, e.g., hippocampus, cer- ebral cortex, and thalamic nuclei. Few viral antigen- or L*-positive cells were detected in the corpus callosum (white matter). At 5 days p.i., the number of viral antigen- or L*-positive cells decreased in tissues of both susceptible and resistant strains. Most of the viral antigen- or L*-positive cells were localized in the gray matter. At 7 days p.i., in both susceptible and resistant strains of mice, a small number of viral antigenpositive cells were identified, although clusters of inflammatory cells were still observed.

Next, double labeling was performed to identify the specific cell type expressing L*. Tissue sections were incubated with primary Ab mixture overnight at 4°C. Sections were incubated with peroxidase-conjugated secondary Ab (Envision+; DAKO), and the bound immunoglobulins (Igs) were detected by 3,3′-diaminobenzidine (tetrahydrochloride; DAB). Then, sections were incubated with alkaline phosphatase-conjugated secondary Ab (Histofine, Nichirei, Tokyo, Japan) and the bound Igs were detected by New Fuchsin (New Fuchsin substrate kit; Nichirei). For double labeling with Abs from the same species, i.e., anti-FLAG MAb/anti-NeuN MAb, sections were incubated with anti-FLAG MAb and were incubated with peroxidase-conjugated secondary Ab. Bound Ig was detected by DAB. Subsequently, sections were incubated with antiNeuN MAb and were incubated with alkaline phosphatase- conjugated secondary Ab. Bound Ig was detected by New Fuchsin. For lectin staining, the sections were incubated with biotinylated lectin and bound lectin was detected with the ABC substrate kit; Nichirei). For double labeling with Abs from the same species, i.e., anti-FLAG MAb/anti-NeuN MAb, sections were incubated with anti-FLAG MAb and were incubated with peroxidase-conjugated secondary Ab. Bound Ig was detected by DAB. Subsequently, sections were incubated with antiNeuN MAb and were incubated with alkaline phosphatase- conjugated secondary Ab. Bound Ig was detected by New Fuchsin. For lectin staining, the sections were incubated with biotinylated lectin and bound lectin was detected with the ABC kit (Vector Laboratories) and DAB. Subsequently, the sections were incubated with anti-FLAG MAb and L* (3xFLAG) was detected by New Fuchsin. Double labeling with anti-FLAG MAb and anti-TMEV Ab showed that L* was colocalized with virus antigen (Fig. 5B). L* and virus antigen double-positive cells had a red-brown color, and there was some difficulty in distinguishing them from the brown cells stained by DAB. In contrast, single-positive cells with red color by New Fuchsin were identified with ease. For further detailed colocalization study, these two antigens were detected by two different staining methods. When L* was detected by New Fuchsin (red) and viral antigen was detected by DAB (brown), L* single-positive cells were not observed. When viral antigen was detected by New Fuchsin and L* was detected by DAB, single-positive (L*-negative) cells were occasionally observed. This indicates that all L*-positive cells express viral antigen but not vice versa. Double labeling with anti-FLAG MAb and cell-specific mark-
ers showed that L* is detectable exclusively in neurons (Fig. 5D). L* was detected in neuronal cell bodies and their processes as well as viral antigens. In contrast, L*-positive cells were not detectable in astrocytes, oligodendrocytes, and macrophages/microglial cells (Fig. 5C, E, and F).

In this study, viral protein and L* were exclusively detected in neurons when wild-type DA or DA/3xFLAGL* mutant viruses were infected. Epitope tagging enabled us to detect L* in both the neuronal cell body and its processes. In early phases of infection by the GDVII and DA strains, GDVII was detected exclusively in neurons (1) (although GDVII can replicate in glial cells [15]), whereas DA was detected not only in neurons but also in astrocytes and possibly in macrophages/microglial cells by in situ hybridization-immunostaining assay (1). These findings are probably due to the sensitivity of detection between the techniques employed for the studies, i.e., viral RNA detection (in situ hybridization) and viral protein detection (immunostaining), but not to the change of virus tropism. The mutant viruses generated in this study have FLAG or 3xFLAG sequence in the viral RNA leader genome,
and L* is translated out of frame with virus polyprotein and not incorporated into the virion (9). Therefore, the mutant viruses have identical capsid proteins with wild-type DA. The molecular sizes of FLAG and 3xFLAG-tags are 1.1 and 2.7 kDa, respectively. The small molecular size is considered to be a great advantage for the investigation of L*; because L* is a relatively small (hydrophobic) 17-kDa protein, it is easier not to disturb its biological functions. Especially, 3xFLAG-tag drastically enhanced the detection of L* both in vitro and in vivo. To demonstrate the presence of L* in vitro, Chen et al. inserted a hemagglutinin (HA) sequence at the XhoI site (nt 1221) in the middle of L* and identified an L*HA fusion protein in BHK-21 cells by immunoblotting (2). HA is also a small epitope tag; however, the XhoI site was located in the middle of L*. Our mutant viruses have epitope tags in the 5′ site of L*, which may be less disturbing to the physiological function of L* in vivo. Besides these epitope tags, green fluorescent protein (GFP) has been used for TMEV studies (20, 21). GFP, the molecular size of which is 26 kDa, is larger than L* itself. Therefore, these small epitope tags may be more useful for investigating L* rather than GFP.

L* is considered to play a key role in viral persistence and demyelination in susceptible strains of mice (2), although its function is controversial (19). This study showed that L* is expressed in both susceptible and resistant strains of mice in the acute phase of infection and the pattern of its expression in susceptible strain is similar to that in the resistant strain. It means that L* expression itself is not a determining factor for susceptibility against DA and that an additional factor or a mechanism interacting with L* is necessary for persistent infection and demyelination. It has been reported that L* has antiapoptotic activity in a macrophage cell line, P388D1 (3). Our recent study showed that L* inhibits the cell death of microglia but not by an antiapoptotic mechanism (11). It has been reported that L* inhibits the generation of H-2K-restricted, virus-specific cytotoxicity in the CNS, permitting a persistent infection in susceptible strains (4). A recent study with the GDVII strain showed that cell-specific expression and differential RNA-binding properties of polypyrimidine tract-binding protein (PTB) and neural-specific PTB are important determinants of cell-specific translational control and viral neurovirulence (12). An increase in the utilization of L* AUG by ribosomes for translation initiation and decreased initiation of translation at the polyprotein’s AUG may limit the production of capsid protein in certain cell types, which are targets for the cytolytic T-cell response. This relative decrease in synthesis not only may prevent the generation of a cytolytic T-cell response but may also favor restricted virus expression. To address this issue, epitope-tagged L* may be useful to quantitate the translation of L*. Additionally, further identification and characterization of L* in the chronic stages of disease await future experiments with mutant viruses expressing small epitope-tagged L*.

This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture and a grant for Project Research from Kanazawa Medical University (S2001-9).

We thank S. Saito for technical assistance.

REFERENCES