Leader (L) of Theiler's murine encephalomyelitis virus (TMEV) is required for virus growth in a murine macrophage-like cell line

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ABSTRACT

Theiler's murine encephalomyelitis virus is divided into two subgroups on the basis of their different biological activities. GDVII subgroup strains cause acute and fatal encephalomyelitis in mice, while TO or DA subgroup strains cause non-fatal polioencephalomyelitis in weanling mice followed by virus persistence and demyelination in the spinal cords. Nonstructural leader (L) protein is encoded at the most N-terminus of the polyprotein. The L coding region of TO or DA subgroup strains has another out-of-frame open reading frame, which produces another nonstructural protein, L*. L* protein is reported to be essential for virus growth in macrophage cells. In the present report, we studied the role of L protein in virus growth in macrophage-like cell line, J774-1, by using a series of deletion mutant viruses. In J774-1 cells (the absence of L* protein), the mutant virus [deleting the entire L coding region (ΔL), N-terminal zinc-finger domain (ΔZ), acidic domain (ΔA), or C-terminal serine/threonine (S/T)-rich domain (ΔS/T)] did not grow. The mutant virus disrupting zinc-finger motif (Lcys) did not grow, either. However, in L* expressing J774-1 cells (the presence of L* protein), Lcys, ΔZ and ΔS/T had a rescue of the growth activity, while ΔL or ΔA had no rescue. The data suggest that L protein is required for virus growth in J774-1 cells and also suggest that the site responsible for virus growth in those cells is the acidic domain of L protein.

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1. Introduction

Theiler's murine encephalomyelitis virus (TMEV) is a single-stranded RNA virus that belongs to the genus Cardiovirus of the family Picornaviridae and is divided into two subgroups on the basis of their different biological activities (Obuchi and Ohara, 1998; Oleszak et al., 2004; Roos, 2002). GDVII subgroup strains cause acute and fatal encephalomyelitis in mice. In the very few surviving mice, no virus persistence or demyelination is observed. In contrast, TO or DA subgroup strains induce an early, non-fatal polioencephalomyelitis of weanling mice followed by virus persistence and chronic demyelination in the spinal cords (Obuchi and Ohara, 1998; Oleszak et al., 2004; Roos, 2002). This late chronic demyelinating disease serves as an experimental model for the human demyelinating disease, multiple sclerosis (Obuchi and Ohara, 1998; Oleszak et al., 2004; Roos, 2002). Studies suggest that macrophages serve as the site of TMEV persistence during the chronic demyelinating phase (Clatch et al., 1990; Lipton et al., 1995). However, the precise mechanisms of virus persistence and demyelination still remain unknown.

Nonstructural protein, L*, is out-of-frame with the viral polyprotein from an alternative AUG, 13 nucleotides downstream from the authentic polyprotein AUG (Kong and Roos, 1991; Obuchi and Ohara, 1998; Roos, 2002). L* protein is only synthesized in DA subgroup strains since the L* AUG is present in DA subgroup strains, but not GDVII subgroup strains (Michiels et al., 1995; Obuchi and Ohara, 1998; Roos, 2002). By both ‘loss of function’ and ‘gain of function’ experiments, L* protein is shown to be essential for virus growth in macrophage cells (Himeda et al., 2005a; Obuchi et al., 1999; Takata et al., 1998), the major site of virus persistence.

On the other hand, another nonstructural protein, leader (L), is encoded at the most N-terminus of the polyprotein (Michiels et al., 1995) and therefore the coding region of L overlaps with that of L*. The L coding region is organized into three domains; an N-terminal atypical (Cys-His-Cys-Cys) zinc-finger domain, an acidic domain, and C-terminal serine/threonine (S/T)-rich domain (Paul and Michiels, 2006). L protein inhibits alpha/beta interferon (IFN) production (van Pesch et al., 2001) at the level of IFN gene transcription by interfering with the nucleocytoplasmic shuttling of IFN regulatory factor-3 (Delhaye et al., 2004). L protein is reported to be required for the assembly of virions (Badshah et al., 2000). It also
regulates viral RNA encapsidation (Takano-Maruyama et al., 2006). Therefore, L protein is a multi-functional protein.

In this study, we explore the possibility that L protein may also contribute to virus growth in macrophage cells, which is a key factor for TMEV persistence. We also clarify the domain responsible for virus growth by generating a series of deletion mutant viruses of those domains.

2. Materials and methods

2.1. Cell culture

BHK21-cells, a baby hamster kidney-derived fibroblast cell line permissive for TMEV infection, were maintained in Eagle’s minimum essential medium (MEM) (Nissui, Tokyo, Japan) supplemented with 0.03% l-glutamine and 5% newborn calf serum (Invitrogen, Carlsbad, CA) containing 60 mg/ml of kanamycin. J774-1 cells were seeded at a density of 3.5 × 10^5 cells in a 35-mm dish. After 24 h, the cells were infected with each virus titrated by a standard plaque assay on BHK-21 cells and the virus growth was examined in the same manner as BHK-21 cells.

2.2. Construction of the mutant cDNAs of DA strain

A series of mutant constructs are depicted in Fig. 1. The detail is described elsewhere (Taniura et al., 2009). Site-directed mutagenesis was used to generate various mutant cDNAs without affecting the amino acid (AA) sequence. pDAFL3 contains a wild-type full-length infectious cDNA from DA strain of TMEV driven under the T7 promoter just upstream from the start of the viral genome (Roos et al., 1997), was used as a template for mutagenesis. To generate a DAAL, in which the entire DA L sequence except AUG initiation codon is completely deleted, two restriction enzyme cleavage sites (NcoI sites at nt 1961 and 6896), followed by the introduction of two restriction enzyme cleavage sites (NcoI sites at nt 1065 and 1291) into the pDAFL3 without affecting the AA sequences. DAL^I is the mutant virus in which two possible L* AUG initiation codons are mutated to ACG to eliminate L* translation.

2.3. In vitro transcription and virus generation

All the plasmid DNAs containing virus genome sequence were linearized with XbaI (TOYOBO, Japan), and RNA transcripts were synthesized with T7 RNA polymerase (Promega, Madison, WI). Then, BHK-21 cells were transfected with the transcripts derived from each construct using Lipofectin (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. The culture cells and supernatants were collected after complete cytopathic effect was observed, and virus lysates were prepared by three freezing/thawing cycles to release virions. Recombinant viruses were purified by a standard plaque purification technique twice and amplified in BHK-21 cells. The titers of recombinant viruses were determined by a standard plaque assay on BHK-21 cells and expressed as plaque-forming units (PFU).

Virus genome was amplified by reverse transcription polymerase chain reaction using total RNA extracted from the infected BHK-21 cells, and confirmed the mutations by sequencing using automated sequencer (Beckman CEQ 8000 Genetic Analysis System).

2.4. Kinetics of virus growth in cells

The kinetics of virus growth of parental and mutant viruses in BHK-21 cells was analyzed as previously described (Obuchi et al., 1997). The cells were seeded at a density of 3.5 × 10^5 cells in a 35-mm dish. After 24 h, the cells were infected with each virus at a multiplicity of infection (MOI) of 5 PFU per cell. After virus adsorption at 37 °C for 60 min, the cells were washed twice with Dulbecco’s phosphate buffered saline (PBS), and incubated at 37 °C in Eagle’s MEM with 1% newborn calf serum up to 48 h. The virus lysates were collected at 0, 3, 6, 12, 24, and 48 h after infection and titrated by a standard plaque assay on BHK-21 cells. In the case of J774-1, L* and control/J774 cells, the cells were seeded at a density of 1 × 10^6 cells in each dish 24 h before virus infection, and the virus growth was examined in the same manner as BHK-21 cells.

2.5. Western blotting

The proteins were extracted from the infected cells with the lysis buffer (10 mM Tris–HCl buffer (pH 8.0), 140 mM NaCl, 3 mM MgCl2, 1 mM DTT, 0.5% NP-40 and 1 mM PMSF), separated by SDS-15% polyacrylamide gel electrophoresis, and transferred onto a polyvinylidene difluoride membrane (Millipore, Billerica, MA). The membrane was blocked with 5% skim milk in PBS-T (PBS containing 0.05% Tween 20) for 30 min and incubated at room temperature for 2 h with anti-L* antibody (Obuchi et al., 2001), followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG for 1 h. Signals were detected using ECL plus Western blotting detection reagents (GE Healthcare Bio-Science) according to the manufacturer’s instructions.
rapidly, reached a peak (3.1 × 10^4 PFU/ml) at 12 h after infection (Fig. 3D). In contrast, the DALcys, DALL viruses in control/J774 cells was similar to the kinetics in J774-1 cells. In L* protein-expressing L'/J774 cells, DAL*-2 virus had a rescue of the growth activity. The titer reached a peak of 7.2 × 10^5 PFU/ml at 12 h after infection (Fig. 3D). In contrast, the DALL mutant virus showed no growth even in L'/J774 cells in which L* protein is expressed (Fig. 3D). These results indicated that not only L* but also L protein is required for virus growth in J774-1 cells.

3.4. Growth kinetics of the domain-deleted mutant viruses in J774-1 cells

To determine the domain responsible for virus growth, we generated domain-deleted mutant viruses (Fig. 1) and examined the growth kinetics of parental DA, DAL*-2 and DAL Delta L mutant viruses in control/J774 cells. As shown in Fig. 5, the growth kinetics of all domain-deleted mutant viruses including DAL Delta L Z, DAL Sigma/T, DAL Sigma/T deleted viruses were severely restricted, and the titers reached a peak of only 1.7 × 10^5 PFU/ml, 5.8 × 10^4 PFU/ml, 7.0 × 10^4 PFU/ml and 1.8 × 10^5 PFU/ml respectively at 12 h after infection (Fig. 4A–D). The level of titers was significantly lower than that of parental DA (2 log to 3 log). As shown in Fig. 5, the growth kinetics of all domain-deleted mutant viruses in control/J774 cells was also similar to the kinetics in J774-1 cells. However, in L'/J774 cells, domain-deleted mutant viruses, DAL Delta L (Fig. 6A), DAL Sigma T (Fig. 6B) and DAL Sigma/T (Fig. 6D), had a rescue of the growth activity, and the viral titers reached a peak of 6.1 × 10^5 PFU/ml, 8.7 × 10^5 PFU/ml and 1.3 × 10^6 PFU/ml respectively at 12 h after infection. The level of virus titers was overall 1–2 log higher than the titers in J774-1 or control/J774 cells. On the other hand, the growth of DAL Delta A mutant virus was severely restricted in L'/J774 cells (Fig. 6C). The peak of the titer in L'/J774 (6.5 × 10^4 PFU/ml) was similar to that in J774-1 cells (7.0 × 10^4 PFU/ml). Therefore, DAL Delta A did not have a rescue of the growth activity at the presence of L* protein, suggesting that the domain responsible for virus growth may be the acidic domain of L protein.

4. Discussion

Recent studies have shown that the TMEV L protein plays a role in host cell-restricted infection. L-deleted DA mutant virus cannot grow in L929 cells, which produce IFN (Kong et al., 1994; van Pesch et al., 2001). Lack of L protein of DA does not affect the virus growth in BHK-21 cells, which are no-IFN-responsive (Calenoff et al., 1995). The L protein inhibits the transcription of IFN-α/β by interfering in the nuclear localization of IFN regulatory factor-3 (Delhaye et al., 2004). The L protein of TMEV is organized into three domains; an N-terminal atypical (Cys-His-Cys-Cys) zinc-finger domain, an acidic domain, and C-terminal Ser/Thr-rich domain (Paul and Michiels, 2004). The L protein encoded from an out-of-frame AUG within the leader coding sequence and was shown to be involved in virus growth in macrophages (Himeda et al., 2005a; Obuchi et al., 1999; Takata et al., 1998; van Eyll and Michiels, 2000). We tested the growth of parental DA, DAL*-2 and DAL Delta L viruses in L* protein-expressing L'/J774 cells (Himeda et al., 2005a). Empty vector-transduced control/J774 cells were used as a control. As shown in Fig. 3C, the growth kinetics of parental DA, DAL*-2 and DAL Delta L viruses in control/J774 cells was similar to the kinetics in J774-1 cells. In L* protein-expressing L'/J774 cells, DAL*-2 virus had a rescue of the growth activity. The titer reached a peak of 7.2 × 10^5 PFU/ml at 12 h after infection (Fig. 3D). In contrast, the DAL Delta L mutant virus showed no growth even in L'/J774 cells in which L* protein is expressed (Fig. 3D). These results indicated that not only L* but also L protein is required for virus growth in J774-1 cells.

3.3. Growth kinetics of parental DA, DAL*-2 and DAL Delta L viruses in L*-expressing J774 cells

Since the L* protein encoded from an out-of-frame AUG within the leader coding sequence and was shown to be involved in virus growth in macrophages (Himeda et al., 2005a; Obuchi et al., 1999; Takata et al., 1998; van Eyll and Michiels, 2000), we tested the growth of parental DA, DAL*-2 and DAL Delta L viruses in the L* protein-expressing L'/J774 cells (Himeda et al., 2005a). Empty vector-transduced control/J774 cells were used as a control. As shown in Fig. 3C, the growth kinetics of parental DA, DAL*-2 and DAL Delta L viruses in control/J774 cells was similar to the kinetics in J774-1 cells. In L* protein-expressing L'/J774 cells, DAL*-2 virus had a rescue of the growth activity. The titer reached a peak of 7.2 × 10^5 PFU/ml at 12 h after infection (Fig. 3D). In contrast, the DAL Delta L mutant virus showed no growth even in L'/J774 cells in which L* protein is expressed (Fig. 3D). These results indicated that not only L* but also L protein is required for virus growth in J774-1 cells.

3. Results

3.1. L* protein expression in virus-infected BHK-21 cells and in L*/J774 cells

First, we examined the L* protein expression in BHK-21 cells infected with parental DA and mutant viruses. BHK-21 cells were infected at 5 MOI per cell, and the cell lysates (20 μg) collected at 6 h after infection were electrophoresed and analyzed by Western blotting. As shown in Fig. 2A, lane 2, a clear 17-kDa protein which was not observed in other lanes, was detected in the cells infected with parental DA virus. Since mutant viruses have an AGC instead of an AUG starting codon at nt 1079 and 1091, L* protein was not detected in mutant viruses-infected BHK-21 cells. As shown in Fig. 2B, lane 3, L* protein was detected in L*/J774 cells although it was not detected in either J774-1 cells (Lane 1) or control/J774 cells (Lane 2).

3.2. Growth kinetics of parental DA, DAL*-2 and DAL Delta L viruses in BHK-21 and J774-1 cells

To determine the functional role of L protein, we examine the growth kinetics of DA, DAL*-2 and DAL Delta L viruses in permissive BHK-21 cells and macrophage-like cell line, J774-1. Fig. 3A showed the growth kinetics of parental DA, DAL*-2 and DAL Delta L mutant viruses in BHK-21 cells. All viruses showed similar growth kinetics; grew rapidly, reached a peak (3.1 × 10^8 to 2.1 × 10^9) at 12–24 h after infection and gradually decreased thereafter. The result indicates that L* and L proteins does not affect the growth of DA strain in BHK-21 cells. We next examined growth kinetics of these three viruses in J774-1 cells. As shown in Fig. 3B, parental DA grew readily, and the titer reached a peak (1.8 × 10^6 PFU/ml) at 6 h after infection and decreased gradually thereafter. In contrast, the DAL*-2 and DAL Delta L mutant viruses showed no growth in J774-1 cells. The titer of these viruses at any time point was low compared with the starting time point. This 'loss of function' experiment using DAL Delta L and DAL*-2 indicates that L* protein is required for virus growth in J774-1 cells. In addition, L protein may be involved in virus growth as well. Furthermore, it is noted that the TMEV yields at 48 h post-infection in J774-1 cells including control/J774 and L*J774 cells are lower than those at the starting time points. Son et al. (2008) reported that the TMEV yields in M1-D macrophage cells steadily decline after 12 h post-infection by apoptosis. Our present data shows the 2 log decrease of parental DA yield at 48 h post-infection in J774 compared with that in BHK-21. This decrease of virus yield may be regulated by apoptosis. In fact, the virus yield of DAL*-2 which does not express anti-apoptotic protein "L*" much more decreased. The deviations of the level of virus yield and the time period when a phenomenon appears may be caused by the different materials used in the experiments (DA/J774 vs. BeAn/M1-D).
Fig. 3. Growth kinetics of parental DA, DAΔL and DAL*-2 viruses. The culture supernatants and cells were harvested at several time points indicated and assayed for titers by a standard plaque assay on BHK-21 cells. Each graph shows the growth kinetics of parental DA (closed circle), DAΔL (open circle) and DAL*-2 (closed reverse triangle) in BHK-21 (A), J774-1 (B), control/J774 (C) and L*/J774 (D) cells. Titers shown are the mean ± S.D. in three independent experiments.

Fig. 4. Growth kinetics of domain-deleted mutant viruses in J774-1 cells. The culture supernatants and cells were harvested at several time points indicated and assayed for titers by a standard plaque assay on BHK-21 cells. The growth curve of each domain-deleted mutant virus was overlaid on those of parental DA (closed circle) and DAΔL mutant virus (open circle). (A) DALΔZ (closed triangle); (B) DAL199 (open triangle); (C) DALΔA (closed square); (D) DALΔS/T (open square). Titers shown are the mean ± S.D. in three independent experiments.
Fig. 5. Growth kinetics of domain-deleted mutant viruses in control/J774 cells. The culture supernatants and cells were harvested at several time points indicated and assayed for titers by a standard plaque assay on BHK-21 cells. Each growth curve of domain-deleted mutant viruses was overlaid on those of parental DA (closed circle) and DAΔL mutant virus (open circle). (A) DALΔZ (closed triangle); (B) DALΔS (open triangle); (C) DALΔA (closed square); (D) DALΔS/T (open square). Titers shown are the mean ± S.D. in three independent experiments.

Fig. 6. Growth kinetics of domain-deleted mutant viruses in L*/J774 cells. The culture supernatants and cells were harvested at several time points indicated and assayed for titers by a standard plaque assay on BHK-21 cells. Each growth curve of domain-deleted mutant viruses was overlaid on those of parental DA (closed circle) and DAΔL mutant virus (open circle). (A) DALΔZ (closed triangle); (B) DALΔS (open triangle); (C) DALΔA (closed square); (D) DALΔS/T (open square). Titers shown are the mean ± S.D. in three independent experiments.
tion, Fan et al. (2009) reported that L protein induces apoptosis in J774-1 cells. It might indicate that there is some additional domain related to apoptosis. In fact, our preliminary data indicate that L protein has an important domain which relates to apoptotic activity in C-terminus.

The present study showed that not only L* but also L plays an important role in virus growth in macrophage cells, which is a major site of TMEV persistence. Therefore, these two nonstructural proteins and the interaction between them will be important key factors to elucidate the pathomechanism(s) of TMEV-induced demyelinating disease.

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Additionally, Fan et al. (2009) reported that L-induced apoptosis is dependent on zinc-finger motif. Based on this report, it was expected that the growth of DALZ is higher than that of DA in J774-1 cells. However, in our study, the growth of DALZ is suppressed in J774-1 cells. The data also demonstrated that L protein is also required for virus growth in J774-1 cells. The present study showed that not only L* but also L plays an important role in virus growth in macrophage cells, which is a major site of TMEV persistence. Therefore, these two nonstructural proteins and the interaction between them will be important key factors to elucidate the pathomechanism(s) of TMEV-induced demyelinating disease.
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