Further characterization and determination of the single amino acid change in the *tsI138* reovirus thermosensitive mutant

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Abstract: Many temperature sensitive mutants have been isolated in early studies of mammalian reovirus. However, the biological properties and nature of the genetic alterations remain incompletely explored for most of these mutants. The mutation harbored by the *tsI138* mutant was already assigned to the L3 gene encoding the λ1 protein. In the present study, this mutant was further studied as a possible tool to establish the role of the putative λ1 enzymatic activities in viral multiplication. It was observed that synthesis of viral proteins is only marginally reduced while it was difficult to recover viral particles at the nonpermissive temperature. A single nucleotide substitution resulting in an amino acid change was found; the position of this amino acid is consistent with a probable defect of inner capsid assembly at the nonpermissive temperature.

Key words: Reovirus, Viral mutant, Thermosensitive mutant, Viral assembly, Sequencing

Résumé: Plusieurs mutants thermosensibles ont été rapidement isolés dès le début de l'étude du réovirus de mammifères. Cependant, les propriétés biologiques et la nature des changements génétiques demeurent peu connues pour la plupart de ces mutants. La mutation au sein du mutant *tsI138* a déjà été localisée comme étant présente sur le gène L3 codant pour la protéine λ1. Dans la présente étude, ce mutant a été étudié davantage comme outil possible pour établir le rôle des potentielles activités enzymatiques de λ1 dans la multiplication virale. Il a été observé que la synthèse des protéines virales est peu affectée alors qu'il est difficile de récupérer des particules virales à température non permissive. La substitution d'un seul nucléotide, entraînant le changement d'un acide aminé, a été retrouvée; la position de cet acide aminé est compatible avec un défaut probable d'assemblage de la capside interne à la température non permissive.

Mots-clés: Réovirus, Murant viral, Mutant thermosensible, Assemblage viral, Séquençage

Introduction

The λ1 protein is one of the major protein of mammalian reovirus inner capsid or core, being found in approximately 120 copies and forming close to 50% of the core mass and 15% of the whole viral particle (Coombs 1998a). Gene reassortment experiments have initially indicated that λ1 is responsible for the temperature optimum and various characteristics of the NTPase activity found in reovirus cores, suggesting that λ1 is actually involved in this activity (Noble and Nibert 1997). Yeast expression of the protein encoded from the cloned gene has further established that the protein can, by itself, exerts NTPase activity *in vitro* (Bisaillon, Bergeron et al. 1997).

The NTPase activity of λ1 appears to be coupled to its ability to unwind double-stranded nucleic acids (Bisaillon, Bergeron et al. 1997), an activity commonly referred to as helicase activity. The presence of a series of consensus motifs such as the classical DEAD box (Bisaillon, Bergeron et al. 1997; Noble and Nibert 1997; Bisaillon and Lemay 1999), and the stronger affinity for single-stranded RNA compared to double-stranded nucleic acids (Bisaillon and Lemay 1997), support the idea of an helicase activity of λ1 during the viral multiplication cycle. The λ1 protein has also been shown to exert an RNA triphosphatase activity on the 5'-triphosphorylated end of nascent mRNAs (Bisaillon and Lemay 1997). This activity could be responsible for the first step in the RNA capping reaction. The higher affinity of λ1 for triphosphorylated 5'-ends on RNA moieties, rather than on free nucleotide (Bisaillon and Lemay 1997), suggest that the helicase activity could be slowed down in the presence of nascent mRNAs, until the RNA triphosphatase activity could act to eliminate the 5' γ-phosphate and release the enzyme to pursue its unwinding activity and allowing transcription to resume.

Interestingly, conditional temperature sensitive mutants have been isolated in the early days of mammalian reovirus discovery but their biological properties and the nature of the genetic changes have been incompletely explored. One such mutant, the *tsI138* mutant, has previously been assigned, using gene reassortment, to the L3 gene encoding λ1 (Ramig and Fields 1979; Ramig, Ahmed et al. 1983) but its biological properties remain poorly studied and genetic changes present were not examined (Coombs 1998b; 2006). In the present study, this mutant was studied as a possible tool to further establish the role of λ1 enzymatic activity in reovirus multiplication. While synthesis of viral proteins was found to be only marginally reduced, the synthesis of genomic RNA was more difficult to assess due to the difficulty in recovering viral particles from cells infected at the nonpermissive temperature. A single amino acid change was found at a position consistent with an effect on inner capsid assembly.

Materials and methods

Cells and viruses

L929 cells were originally obtained from the American Type Culture Collection (ATCC). Wild type reovirus serotype 3 Dearing (T3D) was also obtained from ATCC and propagated at low multiplicity of infection (MOI) on L929. The original inoculum of the *tsI138* thermosensitive mutant of T3D was a generous gift from Dr Kevin M. Coombs (Department of Medical Microbiology and Infectious Diseases, Manitoba University) and was also propagated on L929 cells at the permissive temperature (32°C).

Viral titer and efficiency of plating

Virus titers were determined by determining the 50% tissue culture infectious dose ($TCID_{50}$) on L929 cells at 32°C, essentially as previously described with minor modifications (Danis, Mabrouk et al. 1997). Briefly, tenfold serial dilutions of the virus samples were prepared and used to infect one row (12 wells) of a 96-wells microplate. For each well, a volume of 50µl of virus dilution in serum-free MEM was used. Plates were left at 4°C for one hour before addition of 200µl of complete medium containing 2% of heat-inactivated fetal bovine serum. To determine relative efficiency of plating (EOP 37/32°C), wild type and mutant viruses were similarly processed at 37°C and 32°C except that incubation times were augmented from 6 days at 37°C to 10 days at 32°C.

Efficiency of lysis assay

Rows of 96-wells plates of L929 cells (10^4 cells/well) were infected (in triplicate) with a series of virus dilution to obtain MOI ranging from approximately 40 $TCID_{50}$ units/cell to 0.5 $TCID_{50}$ units/cell. Cells were fixed and stained with methylene blue at 10 days (32°C) or 6 days (37°C) post-infection. Medium was removed, cells were washed briefly by immersing the plate in phosphate-buffered saline, fixed for one hour at room temperature in 4% formaldehyde in PBS. The remaining cells were stained for one hour with 0.5% methylene blue (in PBS) and washed extensively with PBS before being air-dried. The stain was then solubilized with 0.1N HCl (100µl) overnight at 4°C to facilitate visualization; quantitation was done using a plate reader.

Synthesis of viral proteins

L929 cells were infected at an MOI of 10 $TCID_{50}$ units/cell at either the permissive (32°C) or

nonpermissive (37°C) temperature and incubated for different periods of time before metabolic radiolabeling, SDS-PAGE and autoradiography, essentially as previously described (Danis, Mabrouk et al. 1993).

Electron microscopy

L929 cells were infected at an MOI of 10 $TCID_{50}$ units/cell at either the permissive (32°C) or nonpermissive (37°C) temperature and left for either 15 hours (37°C) or 30 hours (32°C) before being fixed and processed, essentially as previously described (Danis, Mabrouk et al. 1993). Samples were processed and analyzed at the electron microscopy facility of INRS-Institut Armand-Frappier (Laval, Québec, Canada).

Sequencing of viral genome L3 segment

Virus stocks were used to infect L929 cells in 10 cm-diameter petri dishes. Cells and medium were recovered at 48 hours post-infection at 32°C, at which time most cells were lysed. Following two cycles of freeze-thaw (-80°C to room temperature), the lysate was extracted once with one-fourth volume of freon (1,1,2-Trichloro-1,2,2,-trifluoroethane, Mallinckrodt Chemicals). After 10 min of centrifugation at 7,500 rpm in a Sorvall SS34 rotor (in Corex 15 ml tubes), supernatant was recovered and overlayed on a 1ml cesium chloride cushion at a density of 1.3 g/ml for ultracentrifugation in a 70Ti rotor at 50,000 rpm for one hour at 4°C. Virus pellet was recovered in 400µl of TEN buffer (10mM Tris-HCl pH 7.5, 1mM EDTA, 250mM NaCl) before addition of 0.5% SDS followed by phenol-chloroform extraction and ethanol precipitation of viral double-stranded RNA. The RNA was denatured at 95°C for 5 min and cooled rapidly on ice. Reverse transcription was done on both strands of the RNA using a pair of primers specific

for each strand of the gene and MLV reverse transcriptase for one hour at 37°C, as recommended by the manufacturer (Roche). One-fifth of the reverse transcriptase reaction was then used in a PCR reaction with the same two oligonucleotides for 35 cycles using FastStart protocol, as recommended by the manufacturer (Roche). The PCR fragment was then purified using Qiaquick PCR purification kit (Qiagen) and sequenced by automated DNA sequencing (Applied Biosystems 3730 DNA Analyzer) in the sequencing service of Institut de Recherche en Immunologie et Cancérologie (IRIC) of Université de Montréal.

Results

Thermosensitivity of reovirus *tsI138*

Although the *tsI138* mutant has been poorly characterized compared to viruses in other complementation groups of reovirus mutants (reviewed by: Coombs 1998b), the relative efficiency of plating (EOP) at 39°C compared to 30°C was reported to be around 10^{-3} while decreasing sharply when higher temperature is used (Coombs 1998b). The relative EOP was thus measured, comparing virus titers at 37°C and 32°C, or alternatively at 37°C and 30°C, using the virus stock propagated in the laboratory from the initial inoculum, as described in Materials and methods. Results obtained varied between 10^{-3} (37°C /32°C) and 10^{-5} (37°C /30°C) and were thus mostly consistent with previous reports using an higher nonpermissive temperature (39°C). Since the virus grows poorly even at the permissive temperature, all the experiments reported herein will compare viral infection at 37°C and 32°C to ensure a certain level of replication while still allowing a good discrimination between the permissive and nonpermissive conditions and minimizing differences in cell growth and kinetic of viral replication for the wild-type virus.

A second approach was undertaken to confirm the thermosensitive phenotype of the virus, namely the efficiency of lysis (EOL) assay (Patrick, Duncan et al 2001). In this procedure, cells are infected at different multiplicity of infection and cell killing at the permissive and nonpermissive temperatures were compared with that of the wild-type virus. Under the conditions used, the wild-type virus was able to kill the cells even more efficiently at 37°C than at 32°C (Fig. 1); even though incubation time was longer at 32°C, this was probably still insufficient to compensate slower viral multiplication at this temperature. In contrast, cell killing with the thermosensitive mutant was very limited at 37°C, even at the highest MOI, while cell killing at 32°C was even better than with the wild-type virus (Fig. 1).

Analysis of reovirus *tsI138* thermosensitive phenotype: protein and RNA synthesis

Synthesis of viral proteins at the permissive and nonpermissive temperatures was next examined in an effort to determine which step(s) in the viral multiplication cycle are blocked at the nonpermissive temperature. Both the wild-type and *tsI138* virus showed a gradual increase in synthesis of viral proteins with time (Fig. 2). For the wild-type virus, the kinetics appear faster at 37°C but a similar level was eventually reached at both temperatures. A similar situation was observed for the *tsI138* virus, although in this case the kinetics appear faster at 32°C than observed with the wild-type. Nevertheless, synthesis of viral proteins does not seem to be significantly affected at the nonpermissive temperature, as judged for very similar levels of the major σ3 outer capsid protein observed at both temperatures 24 hours post-infection and similar peak levels for both viruses at either 37°C or 32°C (Fig. 2). Total accumulation of viral proteins, as measured by immunoblotting, also confirmed that viral proteins accumulation is only slightly decreased in *tsI138*-infected cells at the nonpermissive compared to the permissive temperature

24 hours post-infection (data not shown).

It was previously suggested that the *tsI138* mutant is an RNA-negative mutant i.e. a virus defective in synthesis of the viral double-stranded RNA genome (Coombs 1998b; Coombs 2006). However, efforts to confirm this result were met with little success. While a reduced level of viral dsRNA was observed at the nonpermissive temperature in some experiments, near-normal levels were observed in others (data not shown). This may result from an unstable inner core structure making it difficult to isolate the RNA in a reproducible manner.

Analysis of reovirus *tsI138* thermosensitive phenotype: viral assembly

Altogether, the previous results suggest that a late step in the multiplication cycle of *tsI138* is responsible for a decreased production of infectious virus at the nonpermissive temperature, likely due to either a defect in viral dsRNA synthesis or in viral core assembly. Infected cells were thus examined by electron microscopy in order to determine if a defect in viral assembly can be observed at the nonpermissive temperature (Fig. 3).

At 32°C, numerous double-shelled particles were observed in well-formed viral inclusions for the thermosensitive virus, in wild-type infected cells extensive cytopathic effects were observed with numerous viral particles. The number of completely formed viral particles seemed reduced for the *tsI138*-infected cells, consistent with the fact that the virus is probably still attenuated at this temperature, although this does not appear as a gross defect.

In contrast, at the nonpermissive temperature (37°C), only small, incompletely-formed, inclusions were observed in cells infected with the *tsI138* virus. Structures reminiscent of viral particles were difficult to detect and, as judged by their size and shape, lacked an outer capsid and appeared to be aberrantly assembled (Fig. 3). Our efforts to purify these particles was met with

little success and they were not further analyzed, this probably reflects their unstable nature. There is thus clearly an important defect in the ability to complete viral core assembly at the nonpermissive temperature, consistent with the defect of λ1, as the most abundant? internal core protein. In contrast, the wild-type virus was even more cytolytic at 37°C than at 32°C, and numerous, double-shelled particles, were still clearly observed.

Nucleotide sequence of the L3 gene in reovirus *tsI138*

The nucleotide sequence of the L3, harboring the mutation responsible for the *tsI138* phenotype (Ramig and Fields, 1979) was next determined by RT-PCR amplification on viral genomic RNA using a series of oligonucleotide pairs, as described in Materials and methods. Overall, each nucleotide was read at least twice and the quality of the sequence was verified by direct examination of the chromatograms using the 4Peaks version 1.7.2 software (4 Peaks by A. Griekspoor and Tom Groothuis). Sequences were aligned and compared with sequences of the L3 gene of reovirus serotype 3 Dearing in the NCBI database (accession numbers REOMCPL1A, NC_004274, EF494437 and HM159615) using the CLC Sequence Viewer Version 6.5. Only one nucleotide substitution, a G to T transversion at position 3069, was observed resulting in substitution of a valine at position 1019 for a glycine. This valine is also conserved in all 10 sequences from the 3 main reovirus serotypes available.

Position of Valine 1019 in the crystal structure

The crystal structure of the reovirus core provides information on the location of valine 1019 and its likely importance in viral assembly (Fig. 4A). The outer surface of the λ1 shell is relatively smooth, except for low ridges that border the binding sites of λ2 and σ2. Two

conformers of λ1 are found in the crystal structure that are designated λ1(A) and λ1(B). Comparison of the A and B conformers reveals that two subdomains undergo a simple shift relative to one another. Analysis of the crystal structure reveals that valine 1019 is involved in hydrophobic interactions with residues located on the σ2 protein. For instance, valine 1019 of the λ1(B) subunit is involved in hydrophobic interactions with cysteine 255, leucine 257, and methionine 180 of σ2 (Fig. 4B). Similarly, valine 1019 of λ1(A) is also involved in a hydrophobic interaction with alanine 32 of σ2, although to a lesser extent (Fig. 4C). Analysis of the position of the mutation on the reovirus core structure thus suggests that the substitution of the valine for a glycine at position 1019 could modify the interaction between λ1 and σ2 (Fig. 4).

Discussion

The characterization of the phenotypic properties of the *tsI138* mutant revealed only limited defect upon early steps of the viral replication. It thus seems that the virions that are assembled at the permissive temperature behave normally even when used to infect at the nonpermissive temperature, although the viral titer produced remains low even under permissive conditions**.** The difficulty in reproducible recovery of double-stranded RNA at the nonpermissive temperature suggested a defect in viral core assembly that was confirmed by the limited number, and aberrant morphology, of the viral particles inside the cells, as observed by electron microscopy.

Synthesis of viral proteins appear to be only, at most, marginally reduced at the nonpermissive temperature. One can think that early transcripts are synthesized and translated for a longer time in the absence of adequate core assembly, and this could compensate for the reduced secondary transcription; alternatively, although they appear somewhat unstable, the cores

formed at the nonpermissive temperature may still be able transcribe mRNA at a level sufficient to ensure near-normal level of protein synthesis.

The position of the amino acid substitution on the λ1 crystal structure on the core supports this idea. The substituted amino acid, valine 1019 for a glycine, is found at the external surface of the core structure, rather that in the amino-terminal domain that protrudes inside the viral core (Dryden, Farsetta et al. 1998; Harrison, Farsetta et al. 1999; Reinisch, Nibert et al. 2000). Since it is this amino-terminal domain that harbors the RNA-binding region (Lemay and Danis 1994; Bisaillon and Lemay 1997) and all the consensus motif for the helicase or RNA triphosphatase enzymatic activity (Bisaillon, Bergeron et al. 1997; Noble and Nibert 1997; Bisaillon and Lemay 1999), this suggests that any enzymatic activity exerted by λ1 should not be directly affected by the substitution. In accordance with this idea, preliminary evidence suggests that the protein can bind onto either synthetic dsRNA (polyI-C) or zinc affinity column, as the wild-type protein, even when it was produced at the nonpermissive temperature (data not shown)

Analysis of its position on the core structure suggests that the substitution of the valine for a glycine at position 1019 could reduce the interaction between λ1 and σ2 (Fig. 4). The most likely explanation for the thermosensitive phenotype conferred by the amino acid substitution is thus a defect in core assembly. By analogy, a thermosensitive mutant (*tsE158*) located in the analog protein of avian reovirus, the λA protein was previously reported and also results from a single amino acid substitution (Tran, Xu et al. 2008). In this case, the interaction between λA and λC, the turret protein equivalent to λ2 of mammalian reovirus, is rather affected. The fact that viruses in this complementation group are rarely observed, a single virus was found in both avian and mammalian viruses, suggests the deleterious nature of amino acids changes in these proteins and is consistent with the difficulties of growing the *tsI138* mutant, even at the permissive

285 temperature. These observations suggest that the assembly of the avian λA and mammalian λ1
286 large inner core, is critical during viral morphogenesis.
287

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291

292 References

293 Bergeron, J., Mabrouk, T., Garzon, S. and Lemay, G. 1998. Characterization of the
294 thermosensitive ts453 reovirus mutant: Increased dsRNA binding of sigma 3 protein
295 correlates with interferon resistance. Virology **246**(2): 199-210.
296 doi:10.1006/viro.1998.9188. PMID:9657939.
297 Bisaillon, M., Bergeron, J., and Lemay, G. 1997. Characterization of the nucleoside triphosphate
298 phosphohydrolase and helicase activities of the reovirus lambda1 protein. J. Biol. Chem.
299 **272**(29): 18298-18303. doi:10.1074/jbc.272.29.18298. PMID:9218469.
300 Bisaillon, M. and Lemay, G. 1997. Characterization of the reovirus lambda1 protein RNA 5'-
301 triphosphatase activity. J. Biol. Chem. **272**(47): 29954-29957.
302 doi:10.1074/jbc.272.47.29954. PMID:9368073.
303 Bisaillon, M. and Lemay, G. 1997. Molecular dissection of the reovirus lambda1 protein nucleic
304 acids binding site. Virus Res. **51**(2): 231-237. doi:10.1016/S0168-1702(97)0092-0.
305 PMID:9498620.
306 Bisaillon, M. and Lemay, G. 1999. Computational sequence analysis of mammalian reovirus
307 proteins. Virus Genes **18**(1): 13-37. doi:10.1023/A:1008013117929. PMID:10334035.

308 Coombs, K.M. 1998a. Stoichiometry of reovirus structural proteins in virus, ISVP, and core
309 particles. Virology **243**(1): 218-228. doi:10.1006/viro.1998.9061. PMID:9527931.
310 Coombs, K.M. 1998b. Temperature-sensitive mutants of reovirus. Curr. Top. Microbiol.
311 Immunol. **233**(1): 69-107. PMID:9599922.
312 Coombs, K.M. 2006. Reovirus structure and morphogenesis. Curr. Top. Microbiol. Immunol.
313 **309**: 117-167. doi:10.1007/3-540-30773-7_5. PMID:16909899.
314 Danis, C., Mabrouk, T., Faure, M., and Lemay, G. 1997. Interferon has no protective effect
315 during acute or persistent reovirus infection of mouse SC1 fibroblasts.Virus Res. **51**(2):
316 139-149. doi:10.106/S0168-1702(97)00088-9. PMID:9498612.
317 Danis, C., Mabrouk, T., Garzon, S., and Lemay, G. 1993. Establishment of persistent reovirus
318 infection in SC1 cells: Absence of protein synthesis inhibition and increased level of
319 double-stranded RNA-activated protein kinase. Virus Res. **27**(3): 253-265.
320 doi:10.1016/0168-1702(93)90037-N. PMID:8098176.
321 Dryden, K.A., Farsetta, D.L., Wang, G., Keegan, J.M., Fields, B.N., Baker, T.S. et al., 1998.
322 Internal/structures containing transcriptase-related proteins in top component particles of
323 mammalian orthoreovirus. Virology **245**(1): 33-46. doi:10.1006/viro.1998.9146.
324 PMID:9614865.
325 Harrison, S.J., Farsetta, D.L., Kim, J., Noble, S., Broering, T.J., and Nibert, M.L. 1999.
326 Mammalian reovirus L3 gene sequences and evidence for a distinct amino-terminal region
327 of the lambda1 protein. Virology **258**(1): 54-64. doi:10.1006/viro.1999.9707.
328 PMID:10329567.
329 Lemay, G. and Danis, C. 1994. Reovirus lambda 1 protein: Affinity for double-stranded nucleic
330 acids by a small amino-terminal region of the protein independent from the zinc finger

motif. J. Gen. Virol. **75 (Pt 11)**: 3261-3266. doi:10.1099/0022-1317-75-11-3261. PMID:7964637.

Noble, S. and Nibert, M.L. 1997. Characterization of an ATPase activity in reovirus cores and its genetic association with core-shell protein lambda1. J. Virol. **71**(3): 2182-2191. PMID:9032352.

Patrick, M., Duncan, R., and Coombs, K.M. 2001 Generation and genetic characterization of avian reovirus temperature-sensitive mutants. Virology **284**(1): 113-122. doi:10.1006/viro.2001.0915. PMID:11352672

Ramig, R.F. and Fields, B.N. 1979. Revertants of temperature-sensitive mutants of reovirus: evidence for frequent extragenic suppression. Virology **92**(1): 155-167. doi:10106/0042-6822(79)90221-6. PMID:419688.

Ramig, R.F., Ahmed, R., and Fields, B.N. 1983. A genetic map of reovirus: Assignment of the newly defined mutant groups H, I, and J to genome segments. Virology **125**(2): 299-313. doi:10.1016/0042-6822(83)90203-9. PMID:6836914.

Reinisch, K.M., Nibert, M.L., and Harrison, S.C. 2000. Structure of the reovirus core at 3.6 A resolution. Nature **404**(6781): 960-967. doi:10.1038/35010041. PMID:10801118.

Tran, A.T., Xu, W., Racine, T., Silaghi, D.A., and Coombs, K.M. 2008. Assignment of avian reovirus temperature-sensitive mutant recombination groups E, F, and G to genome segments. Virology **375**(2): 504-513. doi:10.1016/j.virol.2008.02.010. PMID:18353422.

Figure legends

Fig. 1. Relative efficiency of lysis (EOL) of wild-type and *tsI138* virus.

L929 cells were infected in 96-wells plates with either the wild-type or *tsI138* virus at different multiplicity of infection, as indicated, and incubated for either 10 days at 32°C or 6 days at 37°C. Cells were then stained with methylene blue, as described in Materials and methods, and staining quantitated by spectrophotometric measurement using a microplate reader. The percentage or remaining cells (average of three wells) was determined by comparison with mock-infected wells (average of two wells).

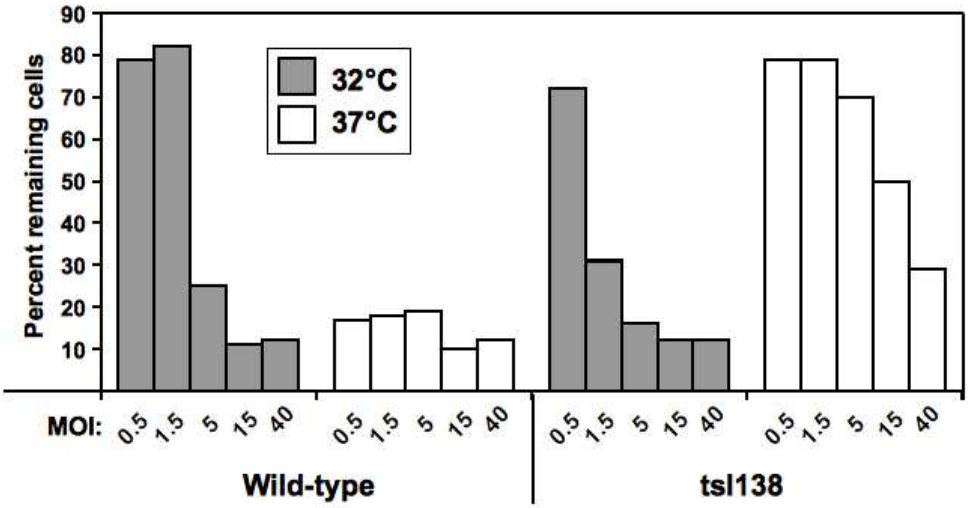
Fig. 2. Synthesis of viral proteins in wild-type and *tsI138* infected cells

L929 cells were infected at either the permissive (32°C) or nonpermissive (37°C) temperature with the wild-type (Wt) or *tsI138* (tsI) virus, as indicated. Metabolic radiolabeling was performed at different times post-infection (15, 24 or 40 hours) and proteins analyzed by SDS-PAGE and autoradiography, as described in Materials and methods. Position of the major outer capsid protein, σ3, is indicated by an arrowhead.

Fig 3. Electron microscopy of infected cells

L929 cells were infected with either wild-type (Wt) or *tsI138* virus (ts) at an MOI of 10 (as determined by the titer obtained by $TCID_{50}$ at 32°C on L929 cells, as described in Materials and Methods, and infected at either the permissive (32°C) or nonpermissive (37°C) temperature. Cells were incubated for 30 hours at 32°C or 15 hours at 37°C before being fixed and processed for electron microscopy, as described in Materials and Methods. A closeup view of the region

373 indicated by a black rectangle is presented in the lower part of the figure, as indicated. Black bar
374 is approximately one micrometer.
375
376 **Fig. 4. Position of the *tsI138* amino acid substitution on the core structure**
377 (A) The position of amino acid 1019 (valine) is presented on both λ1A and λ1B subunits present
378 in the core crystal structure (PDB # 1EJ6) (Reinisch, Nibert et al. 2000). The two subunits of λ1
379 are shown in cyan, the two subunit of σ2 (σ2i and σ2ii) in yellow and the associated λ2 in green.
380 (B and C) Close-up views with emphasis on the residues found in the vicinity of the region
381 surrounding valine 1019 (*WT*). Close-up views are also shown where valine 1019 was replaced
382 by a glycine (*ts*), using the mutagenesis function of PyMol Version 1.4.1 (The PyMOL Molecular
383 Graphics System, Version 1.3, Schrödinger, LLC).



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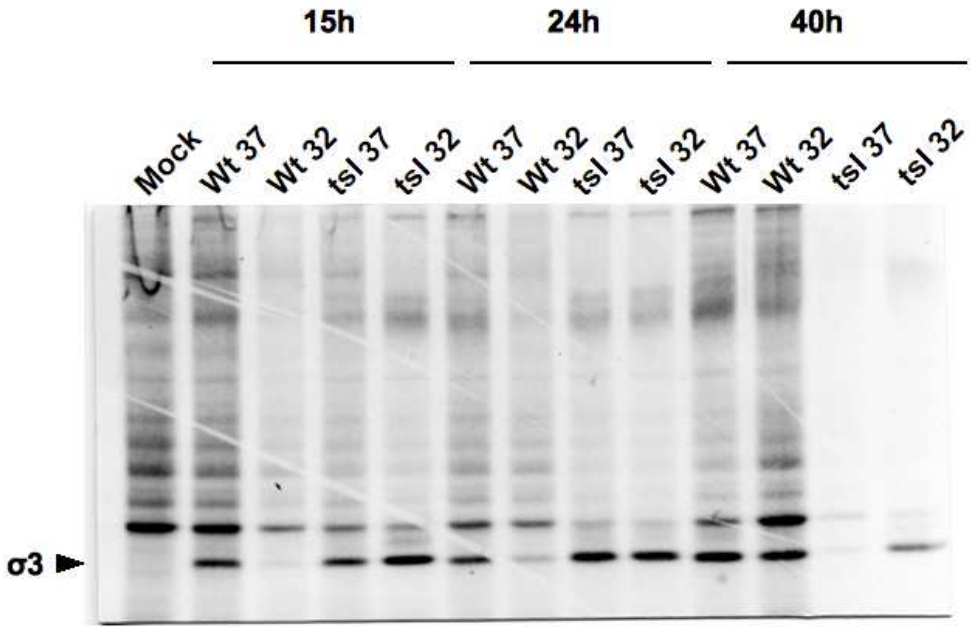

Fig. 2. Synthesis of viral proteins in wild-type and tsIl38 infected cells

L929 cells were infected at either the permissive (32°C) or nonpermissive (37°C) temperature with the wild-type (Wt) or tsI138 (tsI) virus, as indicated. Metabolic radiolabeling was performed at different times post-infection (15, 24 or 40 hours) and proteins analyzed by SDS-PAGE and autoradiography, as described in Materials and methods. Position of the major outer capsid protein, σ3, is indicated by an arrowhead.

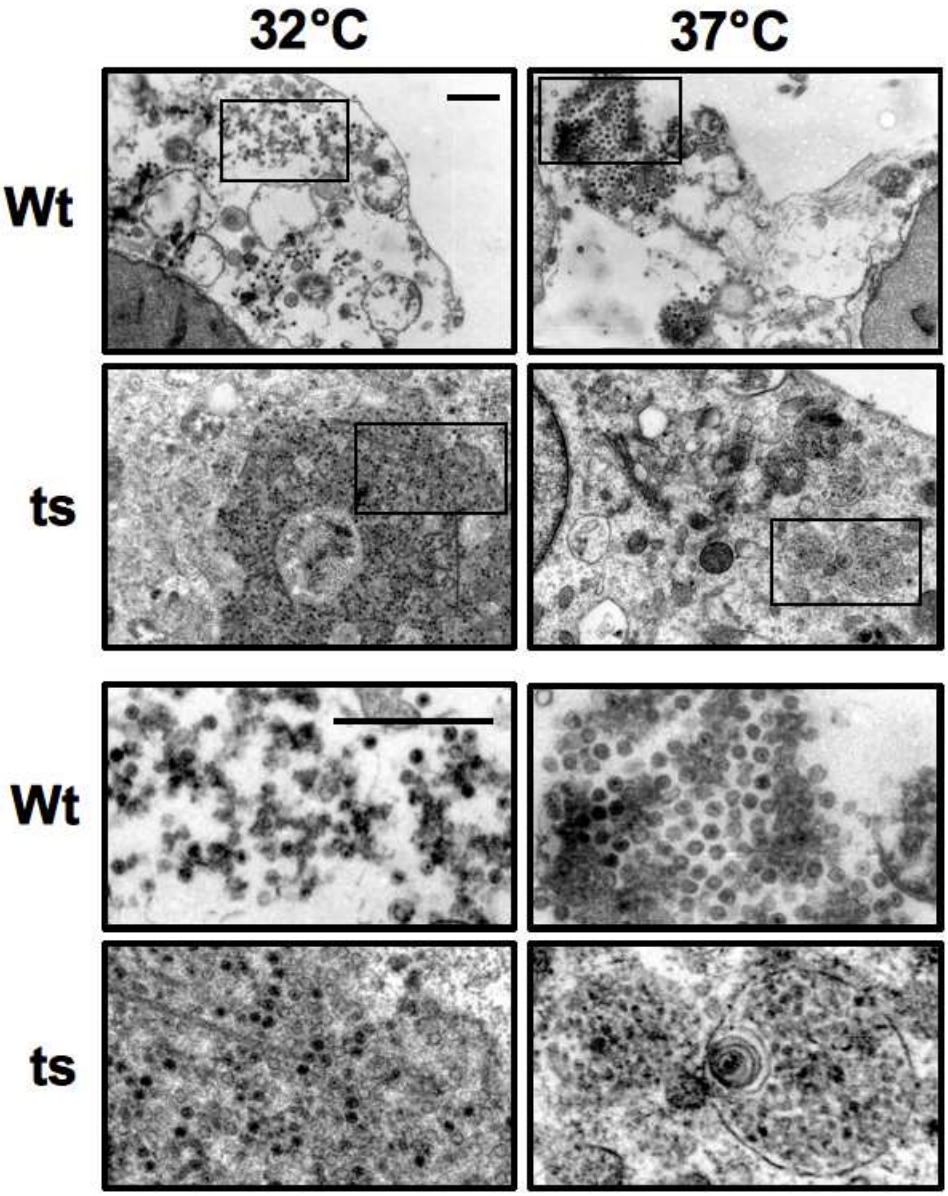

Fig 3. Electron microscopy of infected cells
L929 cells were infected with either wild-type (Wt) or tsI138 virus (ts) at an MOI of 10 (as determined by the titer obtained by TCID50 at 32°C on L929 cells, as described in Materials and Methods, and infected at either the permissive (32°C) or nonpermissive (37°C) temperature. Cells were incubated for 30 hours at 32°C or 15 hours at 37°C before being fixed and processed for electron microscopy, as described in Materials and Methods. A closeup view of the region indicated by a black rectangle is presented in the lower part of the figure, as indicated. Black bar is approximately one micrometer.

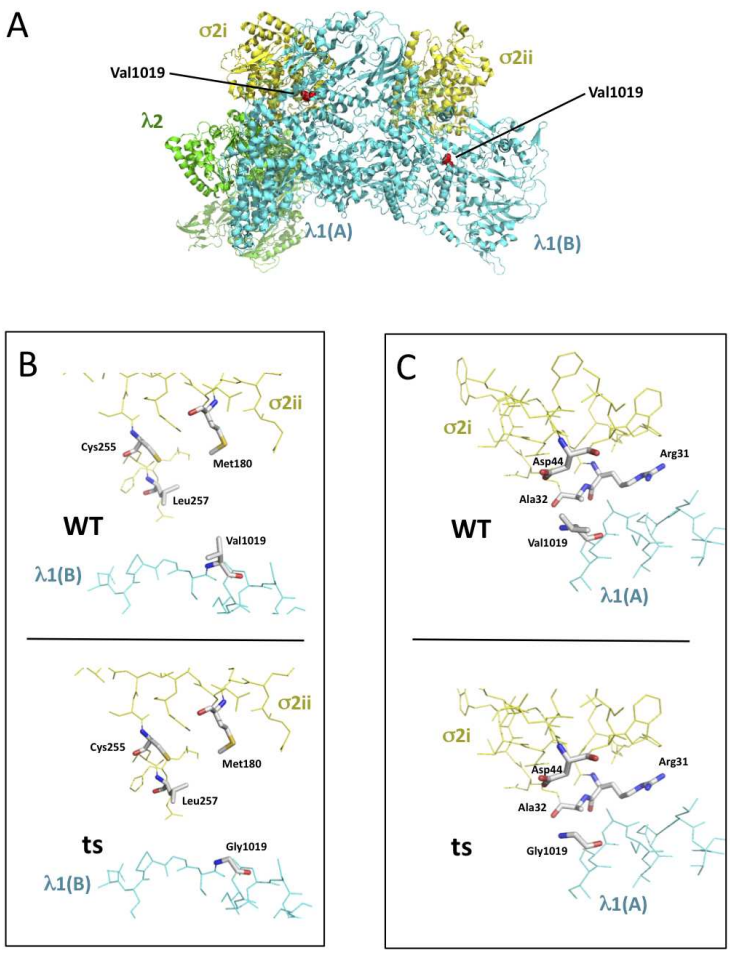

Fig. 4. Position of the tsI138 amino acid substitution on the core structure

(A) The position of amino acid 1019 (valine) is presented on both λ1A and λ1B subunits present in the core crystal structure (PDB # 1EJ6) (Reinisch, Nibert et al. 2000). The two subunits of λ1 are shown in cyan, the two subunit of σ2 (σ2i and σ2ii) in yellow and the associated λ2 in green. (B and C) Close-up views with emphasis on the residues found in the vicinity of the region surrounding valine 1019 (WT). Close-up views are also shown where valine 1019 was replaced by a glycine (ts), using the mutagenesis function of PyMol Version 1.4.1 (The PyMOL Molecular Graphics System, Version 1.3, Schrödinger, LLC).
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