Amino acid mutations in the replicase protein nsP3 of Semliki Forest virus cumulatively affect neurovirulence

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INTRODUCTION

Pathogenesis of Semliki Forest virus (SFV) infection in immunocompetent mice depends on the strain of the virus and the age of the host. Intraperitoneal infection with SFV L10, the prototype SFV strain and SFV4, a cloned derivative, leads to severe encephalitis in mice of all ages (Glasgow et al., 1991; Santagati et al., 1995). Infection with the SFV A7(74) strain is lethal only for mice younger than 2 weeks, whereas in older mice, virus spread is severely restricted in CNS neurons and infection remains asymptomatic (Fazakerley et al., 1993; Oliver et al., 1997); transient demyelination of axons is observed occasionally (Amor et al., 1996; Pathak et al., 1983). In a number of alphaviruses, mutations introduced into the glycoproteins alter virulence; for example, a derivative of virulent SFV4 expressing the A7(74) E2 glycoprotein is apathogenic for adult BALB/c mice. rA774, like several other alphaviruses, has an opal termination codon close to the 5’ end of nsP3 (aa 469), while SFV4 has an arginine residue at this position. Mutation of the opal codon to an arginine residue increases the virulence of rA774 but does not reconstruct the severe neurovirulence of SFV4. Additionally, nsP3 amino acid sequences differ between these two strains by eight amino acids and by a deletion of seven amino acids in the C-terminal third of rA774 nsP3. This study shows that neurovirulence can be reconstituted gradually by exchanging individual amino acids and is fully retained when combinations of two nsP3 mutations, V11→I and L201→F, V11→I and D249→N, A48→E and G70→A or T435→A and F442→L, are introduced into an rA774 derivative carrying R469. The critical role of the arginine codon for neurovirulence was confirmed further by the acquisition of a fully lethal phenotype following the introduction of R469 into a moderately virulent rA774 recombinant carrying the SFV4 nsP1 and nsP2 genes. In conclusion, virulence determinants in SFV are distributed over a wide region of the nonstructural genes.

It has been shown previously that an avirulent Semliki Forest virus (SFV) clone, rA774, engineered to carry the nsP3 gene of the virulent clone SFV4 becomes highly neurovirulent and is lethal for adult BALB/c mice. rA774, like several other alphaviruses, has an opal termination codon close to the 5’ end of nsP3 (aa 469), while SFV4 has an arginine residue at this position. Mutation of the opal codon to an arginine residue increases the virulence of rA774 but does not reconstruct the severe neurovirulence of SFV4. Additionally, nsP3 amino acid sequences differ between these two strains by eight amino acids and by a deletion of seven amino acids in the C-terminal third of rA774 nsP3. This study shows that neurovirulence can be reconstituted gradually by exchanging individual amino acids and is fully retained when combinations of two nsP3 mutations, V11→I and L201→F, V11→I and D249→N, A48→E and G70→A or T435→A and F442→L, are introduced into an rA774 derivative carrying R469. The critical role of the arginine codon for neurovirulence was confirmed further by the acquisition of a fully lethal phenotype following the introduction of R469 into a moderately virulent rA774 recombinant carrying the SFV4 nsP1 and nsP2 genes. In conclusion, virulence determinants in SFV are distributed over a wide region of the nonstructural genes.
may be involved in the inhibition of host cell protein synthesis (Dryga et al., 1997). In contrast, the functions of nsP3, a phosphoprotein (Peränen et al., 1988), are poorly understood. Experiments with cleavage-deficient polyproteins suggest that nsP3 is somehow involved in the generation of cytopathic vacuoles (Salonen et al., 2003). nsP4 is considered to be the RNA polymerase of the virus (Haseloff et al., 1984; Kamer & Argos, 1984). In several alphaviruses, translation of nsP4 requires readthrough of an opal termination codon in the nsP3 gene (Strauss & Strauss, 1994).

We have shown recently that the nsP3 gene of SFV4 can fully restore neurovirulence in adult mice when expressed by rA774. The recombinant virus had a highly pathogenic outcome is milder than that obtained by the exchange of the entire nsP3 gene (Tuittila et al., 2000). Similiar strain-specific opal/sense codon variation is found also in Sindbis and o’nyong-nyong viruses (Simpson et al., 1996; Strauss et al., 1988). Although mutation of the arginie residue to an opal codon dramatically attenuates SFV4, an opal to arginine mutation in rA774 does not reconstitute full neurovirulence, even though it clearly increases pathogenicity (Tuittila et al., 2000). In this study, we show that several combinations of single amino acid mutations of nsP3 can restore lethal neurovirulence. We also show that the opal to arginine mutation in a moderately virulent chimera carrying the nsP1 and nsP2 genes from SFV4 (Tuittila et al., 2000) dramatically increases virulence, whereas only a minor increase in virulence is obtained by the introduction of R<sup>469</sup> into a rA774 chimera carrying the SFV4 structural genes.

**METHODS**

**Cell cultures.** BHK-21 cells (ATCC) were maintained in Glasgow’s minimal essential medium (MEM) supplemented with 5% foetal calf serum (FCS), 5% tryptose phosphate, 2 mM glutamine and 10 mg streptomycin/penicillin l<sup>−1</sup>. MBA-13 cells, transformed mouse brain cells expressing an oligodendrocyte-specific marker (′3′-cyclic nucleotide 3′-phosphodiesterase) were maintained in Eagle’s MEM supplemented with 5% FCS and 10 mg streptomycin/penicillin l<sup>−1</sup>

**Virus strains.** SFV4 was derived from the infectious cDNA clone pSP6-SFV4 (Liljestrom et al., 1991), kindly provided by P. Liljestrom (Karolinska Institute, Stockholm, Sweden). Preparation of prA774, the cDNA clone of A7(74), and its corresponding virus, rA774, has been described previously (Tuittila et al., 2000).

**Construction of RNA expression plasmids.** Construction of prA774-V4nsP3 (SFV4 nsP3 gene and 110 3′-terminal codons of the SFV4 nsP2, including one amino acid mutation, N<sup>217</sup>→S, replacing the corresponding region in rA774), prA774-V4del (rA774 with the 21 nucleotide deletion from rA774 and flanking regions without amino acid mutations inserted from SFV4), prA774-arg (rA774 with nsP3 opal to arginine mutation) and prA774-V4msP12 [rA774 from which the 5′ nontranslated region (NTR), the entire nsP1 gene and the 5′-terminal 689 codons of nsP2 were replaced with the corresponding SFV4 region] has been described previously (Tuittila et al., 2000). The nsP3 regions or full-length genomes of the constructs are shown in Fig. 1. Since the opal (aa 469) to arginine mutation in rA774 was shown previously to be required, but not alone sufficient, to fully restore lethal virulence (Tuittila et al., 2000), rA774, as such, could not be used to study the effect of single amino acid residues. The arginine mutation was introduced into new nsP3 mutants. Additionally, a 327 nucleotide fragment (SacII–NarI, nt 5057–5384) from SFV4 supplying the 21 nucleotides deleted from rA774 was used to replace the corresponding region in mutants. This backbone construct derived from prA774 is designated prA774-V4del-arg, into which V<sup>11→I</sup>, A<sup>48E</sup>→E, G<sup>120A</sup>, L<sup>201→F</sup> and D<sup>249→N</sup> single mutations and V<sup>11→I</sup> in combination with each of the other four mutations were introduced. However, transfections with rA<sup>48E</sup>, rD<sup>249N</sup> and rV<sup>11→A</sup> + A<sup>48E</sup> RNA were nonproductive and therefore A<sup>48E</sup> was combined with G<sup>120A</sup>. Additionally, the V<sup>11→I</sup> mutation was tested in both rA774 and rA774-arg backgrounds (rA774-V<sup>11→I</sup> and rA774-V<sup>11→I</sup>-arg, respectively). Mutations were made with the QuickChange Site-Directed Mutagenesis kit (Stratagene) according to the manufacturer’s instructions. To reveal the contribution of two C-terminal mutations, V<sup>140→E</sup> and F<sup>142→L</sup>, the 3′ end of rA774 nsP3 was replaced with the corresponding part of SFV4 containing the 21 nucleotide insertion, the two mutations and arginine to yield rA774-V4nsP3C. Furthermore, these two mutations were excluded from a construct otherwise identical to rA774-V4nsP3 (Tuittila et al., 2000). This construct was designated rA774-V4nsP3N and the SFV4 originating sequence included 110 3′-terminal codons of the nsP2 and nsP3 genes to the NorI (nt 5384) site downstream from the 21 nucleotide insertion. Additionally, the opal to arginine mutation was included in this construct. rA774-V4nsP12-arg and rA774-V4str-arg (Fig. 1B) were generated by combining rA774-V4nsP12 and rA774-V4str with rA774-arg (Tuittila et al., 2000).

**Sequence analysis.** DNA sequence analysis was performed with a Perkin-Elmer ABI Prism automatic sequencer, model 377.

**Virus production.** Intact full-length cDNA clones were transcribed in vitro using reagents from Roche and the RNA transcripts were electroporated into BHK-21 cells (Bio-Rad gene pulser) (Liljestrom et al., 1995). For large-scale preparation, viruses obtained from the primary cultures were propagated and titrated in MBA-13 cells (Santagati et al., 1995).

**Treatment of mice.** Groups of 10, 4- to 6-week-old female BALB/c AnNHsd mice (Harlan Laboratories) were inoculated intraperitoneally (i.p.) with 10<sup>8</sup> p.f.u. of virus in 100 µl PBS and observed daily for 20 days. Mice were treated and housed at the animal facility of the University of Turku, in accordance with the guidelines of the University Ethics Committee.

**RESULTS AND DISCUSSION**

We have shown previously that replacement of the nsP3 gene in nonpathogenic rA774 with that of neurovirulent SFV4 confers the recombinant lethal phenotype. An important single virulence determinant is an opal codon close to the 3′ terminus of rA774 nsP3. Replacement of the opal codon with SFV4 arginine increases virulence and leads to limb paralysis in a portion of infected mice. However, the outcome is milder than that obtained by the exchange of the entire nsP3 gene. Here we wanted to study the contribution...
to pathogenesis of single nsP3 amino acid residues that differ between rA774 and SFV4. We also studied the role of the opal to arginine mutation in combination with the nsP1 and nsP2 genes as well as the structural genes of SFV4. The nsP3 regions or entire genomes of the recombinants and mutants used in this study are shown in Fig. 1.

The valine residue at nsP3 amino acid position 11 in rA774 was shown to be a PCR mutation that had occurred during the construction of plasmid prA774, as other RT-PCR products obtained later contained an isoleucine codon. Intraperitoneal infection with rA774-V11I, in which the PCR mutation had been corrected, killed 1/10 mice at day 15 post-infection (p.i.) and caused mild paralysis of the hind limb in another mouse, thus appearing slightly more virulent than the parental virus (Fig. 2). In our experiments, mice infected with rA774 or parental SFV A7(74) usually remain completely asymptomatic and only occasionally show paralysis or die (Tuittila et al., 2000). Death following intranasal infection of mice with a related molecular clone CA7 (Tarbatt et al., 1997) has also been observed (G. J. Atkins, personal communication). The outcome of rA774-arg infection was similar to previous studies, where 1/20 mice died and 5/20 were paralysed (Tuittila et al., 2000), although the infection now remained nonlethal and a greater portion of mice (9/10) showed limb paralysis (Fig. 2). A double mutant with the V11→I change and R469 (rA774-V11I-arg) caused mild to severe hind limb paralysis in 6/10 infected mice, of which two died (Fig. 2).

In order to analyse further the contribution of nsP3 single amino acid changes and their combinations to neurovirulence,
we constructed a backbone recombinant, rA774-V4del-arg virus, which now carried the 21 nucleotide insertion, as in SFV4, and an arginine residue in place of the opal termination codon. The insertion was shown previously to have no clinically detectable effect on virulence (Tuittila et al., 2000) but was applied here to allow natural interactions of the amino acid residues of SFV4 nsP3 polypeptide. Substitution of the opal codon with arginine was required to neutralize the attenuating influence of the termination codon. As could be expected, the construct showed increased virulence compared to rA774 (Fig. 2) but was clearly less neuropathogenic than SFV4, leading to the death of only 1/10 mice at 9 days p.i. and causing mild hind limb paralysis in 5/10 mice (Fig. 2). The V11→I mutation in the rA774-V4del-arg backbone (rV11I mutant) led to increased pathogenicity and death of 3/10 mice, while the introduction of the L201F mutation into double mutant rV11I-L201F or the D249N mutation into double mutant rV11I-D249N increased mortality to 100% (Fig. 2). Despite the use of small groups of mice, the increase in mortality of these double mutants in comparison to the rA774-V4del-arg backbone virus or to the single mutant rV11I was statistically significant (Fisher’s exact probability test, P<0.0001 and P<0.002, respectively). Single mutant rL201F killed 4/10 mice, similar to rV11I (Fig. 2). The G70→A mutation led to mild to severe paralysis in 7/10 mice and in combination with the V11→I mutation was lethal for 6/10 mice (Fig. 2), being significantly more lethal than rA774-V4del-arg (P<0.05). Whereas A48→E in combination with V11→I did not yield virus, it was productive together with the G70→A mutation, yielding rA48E+G70A virus, which was highly virulent and killed 10/10 mice in 8 days and was thus comparable to the double mutants rV11I-L201F and rV11I-D249N (Fig. 2). Constructs with the nsP3 changes A48→E or D249→N did not yield infectious virus. The reason for the nonviability of the three constructs remains obscure, as DNA sequencing revealed no additional mutations in the regions subjected to in vitro mutagenesis. Also, the cloning steps were repeated but no virus was obtained. It is, however, possible that these mutations were incompatible with the rest of the genome. Nonviable chimeras have also been created from the cDNA of

**Fig. 2.** Kinetics of mortality and clinical symptoms in SFV infection. Female 4- to 6-week-old BALB/c AnNHsd mice were infected i.p. with 10⁶ p.f.u. of virus in 100 μl PBS. Bars indicate the number of surviving mice. White, asymptomatic; grey, slightly paralysed; black, severely paralysed.
neurovirological and avirulent Sindbis virus (Simpson et al., 1996).

Recombinant rA774-V4nsP3N expressing all but the two C-terminal nsP3 mutations killed 10/10 animals, corresponding to rA774-V4nsP3 virus with a complete SFV4 nsP3 gene (Fig. 2). Both infections showed similar kinetics, with mean time of death being 6-4 days p.i. (Fig. 1). Unexpectedly, the introduction into rA774 of the C-terminal third of SFV4 nsP3 containing the mutations T435→A and F442→L together with the seven amino acid insertion and R469 dramatically increased virulence. The resulting virus rA774-V4nsP3C killed 8/10 mice within 8 days and induced severe paralysis in both survivors (Fig. 2).

Although the nsP3 gene was shown to be an important pathogenicity determinant, it is not the only virulence factor. We have shown previously that recombinant rA774-V4nsP12, in which the 3' NTR, nsP1 and nearly the entire nsP2 gene of rA774 have been replaced with the corresponding region of SFV4, is moderately virulent, showing 35% mortality in mice despite the presence of the nsP3 opal codon (Tuittila et al., 2000). This was in conflict with the strict reduction in mortality achieved by the replacement of R469 in SFV4 with an opal codon (Tuittila et al., 2000). Introduction of R469 to rA774-V4nsP12 increased lethality to 100%, with a mean time of death of 4-2 days p.i. (Figs 1 and 2). Control infection with rA774-V4nsP12 killed 3/10 mice and caused paralysis for 4/10 infected mice, thereby reproducing previous results. On the other hand, the introduction of R469 to the avirulent construct expressing rA774 genes for nonstructural proteins in combination with SFV4 structural protein genes yielded a virus that was clearly less virulent than rA774-arg. This virus, designated rA774-V4str-arg, induced transient, mild hind limb paralysis in 1/10 mice (Fig. 2). This result suggests that the structural genes of virulent SFV4 are less competent in the production of severe neuropathological disease than those of avirulent rA774. Therefore, the low virulence of V4-opal in comparison to the moderate virulence of rA774-V4nsP12 (Tuittila et al., 2000) may be explained by the relatively inefficient structural genes of V4-opal. Also, the virulence of SFV4 and SFV prototype is reduced as nsP3-opal. Whereas the functions of nsP3 are poorly understood, several functional domains or active sites have been localized in nsP1 and nsP2 (Fig. 3). In the N-terminal half of nsP1 resides the conserved methytransferase/capping enzyme domain, the N-terminal part of which evidently binds to S-adenosylmethionine (AdoMet) during capping reactions (Ahola et al., 1997). SFV4 and L10 strains differ from rA774 by only one amino acid in the conserved domain. rA774 has a serine residue at position 237, whereas virulent strains have a cysteine residue (Fig. 3). Membrane association of nsP1 is required for capping enzyme activity (Ahola et al., 1999) and prevention of palmitoylation of cysteine residues 418–420 by mutagenesis renders the virus avirulent (Ahola et al., 2000). However, in rA774, both the membrane-binding segment (Lampio et al., 2000) and the palmitoylation site (Laakkonen et al., 1996) are identical to the other SFV strains (Fig. 3).

nsP2 can be divided into the N-terminal helicase domain and the C-terminal protease domain (Gomez de Cedron et al., 1999; Gorbalenya et al., 1991; Vasiljeva et al., 2001). Mutation K192→N in the helicase domain abolishes nucleoside triphosphatase and helicase activities as well as RNA 5'-triphosphatase activity involved in capping reactions (Gomez de Cedron et al., 1999; Rikkonen et al., 1994; Vasiljeva et al., 2000). The protease domain contains the nucleolar targeting region and the nuclear localization signal (PRRRV651) (Rikkonen et al., 1992). Whereas SFV4 only differs by four amino acid residues from rA774 in nsP2, the other strains, CA7, SFV prototype and L10, exhibit several different residues (Fig. 3).

The N-terminal two-thirds of nsP3 are conserved among alphaviruses, while the C-terminal part is hypervariable and large deletions and insertions are well tolerated there (Lastarza et al., 1994a). Instead, insertions in the conserved region of Sindbis virus nsP3 affect synthesis of minus-strand and subgenomic RNA (Lastarza et al., 1994b). Amino acids 18–115 of nsP3 comprise a conserved region known as the X domain. It is found in animal but not plant viruses of the alphavirus-like superfamily, including Rubella virus and hepatitis E virus (van der Heijden & Bol, 2002). Also, otherwise unrelated coronaviruses possess this domain (Gorbalenya et al., 1991; van der Heijden & Bol, 2002) and identity is found even with the nonhistone part of macrohistone mH2A1.1 of eukaryotes as well as with bacterial proteins (Pehrson & Reina, 1998). The function of the X domain is presently unknown. It is always associated with papain-like proteinases (Gorbalenya et al., 1991) and in Rubella virus it is important for polyprotein cleavages in trans but not in cis (Liang et al., 2000). In mammals, mH2A1 is associated preferentially with inactive X chromosomes, probably participating in transcriptional silencing in interaction with Xist RNA (Costanzo & Pehrson, 1998; Csankovszki et al., 1999).

cDNA clone CA7 (Tarbatt et al., 1997) shares with rA774 only two nsP3 mutations, SFV4 glutamic acid to alanine (aa 48) and SFV4 alanine to glycine (aa 70), while the other
mutations, including the opal codon and the deletion of seven residues, are unique for rA774 (Tuittila et al., 2000). On the other hand, CA7 has several mutations that are not found in rA774 (Fig. 3) and the apathogenicity of CA7 derives from mutations in both structural and nonstructural proteins (Tarbatt et al., 1997).

Comparison to other alphaviruses (Fig. 4) revealed conservation of nsP3 Ile^{11}, which, during construction of rA774, was changed to valine. Back-mutation produced a virus that killed 1/10 mice. The mutation is close to the site where nsP2/3 is cleaved by nsP2 protease activity for formation of the stable replication complex and cessation of minus-strand RNA synthesis (Lemm et al., 1994). The amino acids needed for cleavage site recognition are not known. Although, the similarity of isoleucine and valine suggests toleration of the mutation, differences in polyprotein processing could change the rate of replication and affect pathogenesis. The A^{48}→E mutation in combination with the nonlethal G^{70}→A change produced a lethal virus, killing 100% of infected mice. The lethal phenotype was totally unexpected because E^{48} was found only in SFV4, whereas alanine was present both in rA774 and virulent SFV prototype and L10 strains as well as in some other viruses of the SFV antigenic complex. The glycine residue at position 70 was shared by all other alphaviruses, except SFV4, SFV prototype and L10, which all had alanine residue, another small and nonpolar residue, at this position. Both the conservation of G^{70} and its similarity to alanine are in accordance with the observation that this mutation had only little effect on virulence. Infections with the double mutants rV^{11}I+L^{201}F and rV^{11}I+D^{249}N were lethal for mice. F^{201} and N^{249} were shared by CA7 clone and the virulent strains of SFV, but L^{201} and D^{249} of rA774 were more common among other alphaviruses. The mutations T^{435}→A and F^{442}→L reside in the C-terminal hypervariable region of nsP3 and thus sequences around them could not be compared reliably with other alphaviruses, but within SFV strains, T^{435} and F^{442} were unique for rA774. The strong impact on virulence of these mutations in a region where even a deletion of seven amino acids does not affect the outcome of infection was unexpected. The hypervariable region also includes serine and threonine residues within aa 320–367, which are post-translationally modified by phosphorylation (Vihinen et al., 2001; Vihinen & Saarinen, 2000), but this region is identical in rA774 and SFV4.

In infected BHK-21 cells, SFV nsP3 proteins are located on cytopathic vacuoles (Peränen & Kääriäinen, 1991), the sites of RNA replication (Froshauer et al., 1988), or distributed in the cytoplasm. In a study with cleavage-deficient polyproteins,
nsP123 was shown to be associated with cytopathic vacuole-like structures via nsP1 but the presence of nsP3 in the polyprotein was required for formation of the vacuoles. When expressed alone in mammalian cells, nsP3 molecules form loose aggregates (Salonen et al., 2003).

Therefore, it was no surprise that the nsP3 proteins of rA774 and SFV4, expressed alone using a vaccinia expression system, exhibit different localization in HeLa cells (data not shown). Neither could we show any difference in the distribution of nsP3 in BHK-21 cells infected with rA774, SFV4 or different recombinants and mutants (data not shown). This does not, however, exclude possible differences in the distribution of nsP3 in mature CNS neurons in which replication of A7(74) is restricted (Fazakerley et al., 1993).

The block of A7(74) replication in mature neurons is suggested to occur post-translationally because capsid proteins containing viral core aggregates but no mature virions are detected in the neurons of adult mice (Fazakerley et al., 1993). Treatment of adult mice with gold sodium thiomalate, which stimulates smooth membrane synthesis in CNS neurons, renders A7(74) infection lethal (Mehta et al., 1990). In the brains of gold sodium thiomalate-treated, A7(74)-infected mice, neuronal processes stained positive for capsid protein, whereas without thiomalate pre-treatment, only cell bodies of A7(74)-infected neurons were positive for capsid protein (Scallan & Fazakerley, 1999). During post-natal axono- and synaptogenesis when endo- and exocytosis are active in the murine olfactory system, A7(74) spreads interneuronally and infection is lethal for young mice, unlike that for adult animals (Oliver & Fazakerley, 1998). The nonstructural proteins are associated with cytopathic vacuoles. This way, they could be involved in activating membrane traffic and might have influence in even later steps of the virus life cycle, including assembly and budding.

The fact that single amino acid changes and, more pronounced, their combinations at different locations of nsP3 dramatically increased virulence suggests that the structure of nsP3 plays an important role in replication and is very sensitive to minor changes. Thus, minor changes can essentially alter the protein surface hydrophobicity and, given the hypothetical vacuole-inducing function of nsP123, the surface structure probably represents a crucial parameter for this interaction.

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