

A single mutation in the mammalian orthoreovirus S1 gene is responsible for increased interferon sensitivity in a virus mutant selected in Vero cells

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ABSTRACT

In a previous study, a mammalian orthoreovirus mutant was isolated based on its increased ability to infect interferon-defective Vero cells and was referred to as Vero-cells-adapted virus (VeroAV). This virus exhibits reduced ability to resist the antiviral effect of interferon. In the present study, the complete genome sequence of VeroAV was first determined. Reverse genetics was then used to identify a unique mutation on the S1 gene, overlapping the $\sigma 1$ and $\sigma 1s$ reading frame, resulting in increased sensitivity to interferon. A virus lacking $\sigma 1s$ expression consecutive to mutation of its initiation codon was then shown to exhibit a further increase in sensitivity to interferon, supporting the idea that $\sigma 1s$ is the viral protein responsible. This identification of a new determinant of reovirus sensitivity to interferon gives credentials to the idea that multiple reovirus genes are responsible for the level of interferon induction and susceptibility to the interferon-induced antiviral activities.

1. Introduction

In the last few years, it has been shown that multiple virus genes are often involved in the control of the innate immune response mediated by the interferon signaling network (Fensterl et al., 2015; Beachboard and Horner, 2016; Schulz and Mossman, 2016; García-Sastre, 2017). Different viruses were also shown to exhibit many sequence changes when grown under conditions where the selective pressure to keep viral moderators of the interferon response is removed (Perez-Cidoncha et al., 2014; Hernández-Alonso et al., 2015; Garijo et al., 2016; Weber-Gerlach and Weber, 2016; Du et al., 2018).

The interferon response is well known to be of critical importance as a determinant of viral pathogenicity and a better understanding of viral determinants involved is thus fundamental for epidemiological surveillance as well as choosing the appropriate treatment option. It is also well accepted that the interferon response often plays a role in determining the ability of a virus to discriminate between parental and transformed/cancer cells; the latter frequently, but not always, exhibiting a reduced interferon response (Randall and Goodbourn, 2008; Naik and Russell, 2009; Katsoulidis et al., 2010; Kaufman et al., 2015; Ebrahimi et al., 2017; Matveeva and Chumakov, 2018). Since a plethora of viruses are presently considered as possible oncolytic viruses for cancer treatment (Ilkow et al., 2014; Miest and Cattaneo, 2014; Pikor et al., 2015; Turnbull et al., 2015), this aspect could be of

importance. A better understanding could lead to the selection of better optimized and appropriate virus strains for given cancer cell types (Pikor et al., 2015; Sanjuán and Grdzlishvili, 2015).

Mammalian orthoreovirus (herein referred to as reovirus) is among the viruses presently under study for their possible use as anticancer agents; it presents the advantage of exhibiting a natural tropism for cancer cells (Black and Morris, 2012; Maitra et al., 2012; Clements et al., 2014; Chakrabarty et al., 2015). The interferon response does appear to be at least partly involved in the oncoselectivity of reovirus (Strong et al., 1998; Rudd and Lemay, 2005; Shmulevitz et al., 2010). Another advantage of reovirus as a putative agent in virotherapy is that it is believed to be essentially nonpathogenic in humans. However, this does not preclude the possible emergence of novel viral strains of increased pathogenicity, as suggested in the last few years (Kohl and Kurth, 2015; Thimmasandra Narayanappa et al., 2015; Yang et al., 2015). The importance of the interferon response in viral pathogenicity does not need to be further stressed. This is also the case in reovirus whose pathogenicity in the heart and brain of animal models is clearly linked to its ability to control, or not, the interferon response (Sherry et al., 1998; Goody et al., 2007; Zurney et al., 2007; Tyler et al., 2010; Dionne et al., 2011; Irvin et al., 2012; Wu et al., 2018). Also, a recent study has raised the possibility that the virus contributes to the development of celiac disease in humans and, once again, there is a likely possibility that the interferon-mediated immune response is involved

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(Bouziat et al., 2017).

It thus appears to be essential to gain a further understanding of the viral determinants that control induction of the interferon response and the sensitivity of different viral isolates to this response. This is one of many approaches that could lead to next-generation reovirus variants that will be either more potent or more specific in their oncolytic effect (Mohamed et al., 2015; Kemp et al., 2016). In this context, a reovirus mutant was previously obtained by the establishment of persistence and thus long-term growth of the virus in Vero cells (Jabre et al., 2013; Sandekian and Lemay, 2015b). These cells are known to be deficient in production of interferon, interferon regulatory factor 3 (IRF3) and interferon-induced protein kinase PKR (Desmyter et al., 1968; Emeny and Morgan, 1979; Park et al., 2006; Chew et al., 2009). As somewhat expected, the resulting Vero-cell-adapted virus (VeroAV) was both adapted for better growth in Vero cells and exhibited an increased sensitivity to interferon compared to the original wild-type virus (Jabre et al., 2013; Sandekian and Lemay, 2015b); however, this last aspect was not examined in detail.

In the present study, the complete genome sequence of VeroAV was first determined and revealed actual amino acids substitutions in four virus genes. The plasmid-based reverse genetics (Kobayashi et al., 2007, 2010) was then applied to introduce each of these mutant genes in the original wild-type interferon-resistant genetic background. This allowed to confirm a strongly increased interferon sensitivity with no difference in interferon secretion and the phenotype was assigned to the S1 gene that harbors two separate mutations. The mutation responsible for increased interferon sensitivity was then shown to be located in the region of the S1 gene corresponding to the amino-proximal region of $\sigma 1$ where its reading frame overlap with that of $\sigma 1s$. Introduction of a stop codon in the $\sigma 1s$ -encoding reading frame of an otherwise wild-type virus also increases interferon sensitivity indicating that $\sigma 1s$ is the most likely determinant involved in the virus mutant. The $\sigma 1s$ protein thus needs to be added to the many reovirus proteins shown to be involved in the control of either induction and/or sensitivity to the interferon response.

2. Results

2.1. Sequencing of the VeroAV genome

To determine the differences in sequence between the Vero-cell-adapted virus (Jabre et al., 2013; Sandekian and Lemay, 2015b) and the original serotype 3 Dearing (T3/Human/Ohio/Dearing/55) laboratory strain (T3D^S), the whole coding sequence of the genome was obtained. Reverse transcription-PCR on the semi-purified viral genome from both the mutant and wild-type laboratory stocks was used to generate PCR products that were directly subjected to sequencing, as described in Materials and Methods and previously used in the laboratory. In a previous study, the sequence of S1 and M2 was solely reported (Sandekian and Lemay, 2015b).

Overall, four genes were found to exhibit nonsynonymous mutations compared to the wild-type resulting in a total of 7 amino acids substitutions on 5 proteins since one of the two mutations overlaps the two reading frames in S1 (Table 1). Only three additional silent nucleotide substitutions were found between the wild-type and VeroAV (data not shown). All sequences were deposited in GenBank: the wild-type T3D^S sequences were previously deposited under accession numbers KP208804 to KP208813 and sequences for VeroAV were added in the present study under accession numbers MK238541, MK238542 and MK246415 to MK246422.

2.2. Reverse genetics rescue and characterization of VeroAV

To confirm that the amino acid substitutions in the four VeroAV genes (S1, M1, M2 and M3) are actually responsible for the difference in interferon sensitivity between the two viruses, they were introduced in

Table 1

Amino acids differences between wild-type reovirus type 3 Dearing laboratory stock (T3D^S) and derived Vero-cell-adapted virus (VeroAV).

Protein(gene)	Wild-type (T3D ^S)	VeroAV	Amino acid position
$\mu 2$ (M1)	G	R	389
$\mu 1$ (M2)	E	G	89
	A	V	114
μ NS(M3)	D	N	706
$\sigma 1$ (S1)	Q	P	78
	N	K	198
$\sigma 1s$ (S1)	N	H	59

the T3D^S background made of the other 6 genes. Cells were infected with the resulting virus and supernatants recovered to measure secreted β and α -interferons, as described in Materials and methods.

The rescued VeroAV induced similarly low amount of interferon as the control rescued wild-type, T3D^S (Fig. 1). This is consistent with previous observations using a less direct approach where the ability of infected cells supernatants from the original T3D^S and VeroAV were shown to exhibit a similar ability to protect against another virus (Jabre et al., 2013).

Interferon sensitivity was then examined first by the decrease of the virus titer in the presence of a near-saturating (200 international units [IU] per ml) concentration of type I interferon (mouse β -interferon without carrier protein, PBL Assay Science). As previously shown (Sandekian and Lemay, 2015a; Lanoie and Lemay, 2018), the rescued T3D^S was quite resistant under these conditions. In contrast, the rescued VeroAV was essentially a hundredfold more sensitive (Fig. 2), as previously observed with the original VeroAV (Jabre et al., 2013). Secondly, the sensitivity to different concentrations of interferon was determined as described in Materials and methods and as used before (Sandekian and Lemay, 2015a; Lanoie and Lemay, 2018). Briefly, consecutive twofold dilutions of interferon were prepared from 250 IU/ml to approximately 4 IU/ml in single wells of a microplate; cell lysis in each well (4–5 days post-infection at a MOI of 0.05) was measured by transparency following methylene blue-staining compared with control well containing mock-infected cells. Again, the rescued VeroAV was significantly more sensitive than the wild-type T3D^S (Fig. 3); using this approach, the concentration of interferon needed to decrease cell lysis from 100% (in the absence of interferon) to 50% was more than 250 IU/ml for T3D^S but less than 2.5 IU/ml for VeroAV.

2.3. Introduction of different VeroAV genes in the T3D^S background

Having confirmed that the four genes are actually sufficient to increase sensitivity to interferon, the three genes M1, M2 and M3 of VeroAV were then separately introduced in the wild-type T3D^S background from which VeroAV was originally derived. In addition, a combination of both M2 and S1 of VeroAV was also introduced in the same background. This was necessary to examine the effect of S1 since it was previously shown that this mutant gene is poorly compatible with the wild-type M2 gene thus rendering analysis impossible due to strikingly reduced viral titer and virus replication (Sandekian and Lemay, 2015b).

The introduction of either the M1, M2 or M3 gene by themselves had no effect on the interferon sensitivity using either the reduction of titer assay or the interferon dilution assay (Fig. 2 and Fig. 3 upper panel). In contrast the introduction of S1 (with M2) resulted in a virus as sensitive as VeroAV (Fig. 2 and Fig. 3 upper panel), indicating that the S1 gene is the only determinant of this sensitivity.

2.4. Introduction of single mutations in the S1 gene

Having established that the S1 gene is responsible for the increased interferon sensitivity of VeroAV, each of the two mutations on this gene

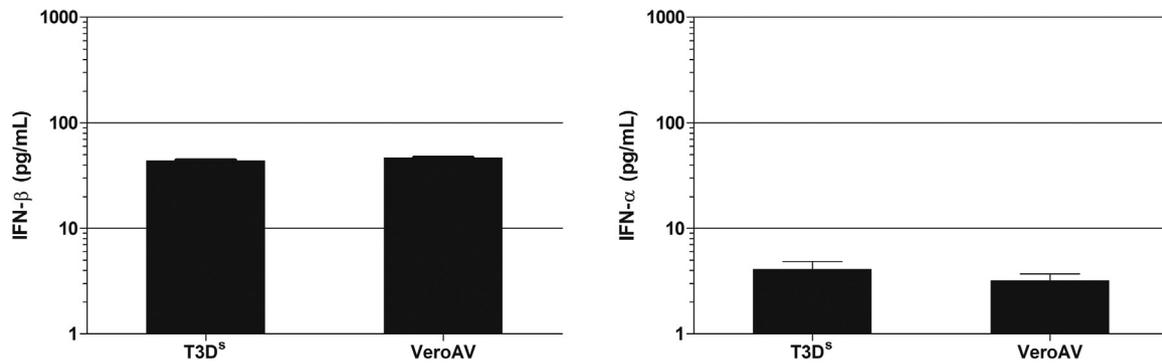


Fig. 1. Comparison of interferon induction by the two rescued viruses. L929 cells were infected at a MOI of 20 TCID₅₀ units per cell with either the rescued parental wild-type T3D^S or the rescued VeroAV. Infected cells media were recovered at 15 h post-infection and directly used in commercial ELISA assays, as described in Materials and methods. Quantitation of secreted β -interferon or α -interferons are presented, as indicated. Results are shown as the average of two independent experiments with error bars representing the standard error of the mean. The differences between T3D^S and VeroAV were not statistically significant.

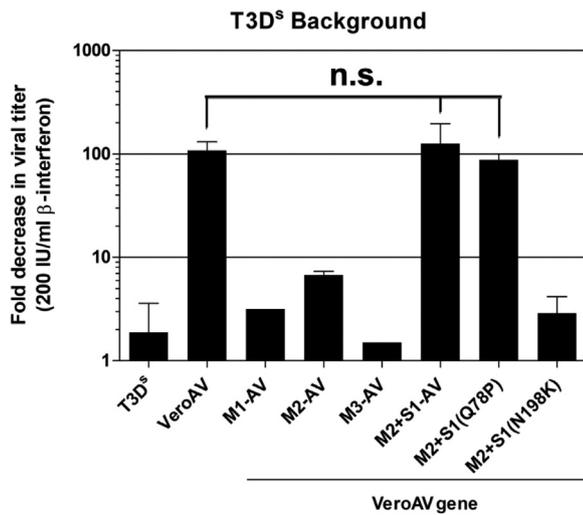


Fig. 2. Interferon sensitivity of T3D^S harboring VeroAV genes. VeroAV genes were separately introduced in the T3D^S background, except for the S1 gene that was introduced in combination with M2. Also, VeroAV S1 gene harboring either one of the two mutations corresponding to Q78P or N198K substitutions in $\sigma 1$ were separately introduced in the T3D^S background in combination with VeroAV M2 gene. Virus titers in the absence or presence of 200 IU/ml of β -interferon were compared, as described in the text. Fold reductions in titers due to interferon presence are presented on a logarithmic scale. Error bars indicate the highest value obtained in two or more experiments. The two viruses that are not significantly different from VeroAV, using Student's *t*-test are indicated ($p > 0.2$; n.s.), while others are significantly different ($p < 0.01$).

(resulting in Q78P and N198K amino acids substitutions in $\sigma 1$) were solely introduced. When interferon sensitivity was assessed, the Q78P substitution was found to be both necessary and sufficient to fully reconstitute the interferon sensitivity of VeroAV (Fig. 2 and Fig. 3 lower panel).

The involvement in interferon sensitivity of the virion binding region of the viral attachment protein appears unlikely. However, the mutation resulting in Q78P substitution on $\sigma 1$ also overlaps the reading frame for the small nonstructural $\sigma 1$ s in S1, the Q78P substitution in $\sigma 1$ thus also results in a N59H substitution in $\sigma 1$ s.

2.5. Effect of $\sigma 1$ s on interferon sensitivity

The previous data indicate that the amino acid substitution in the small $\sigma 1$ s protein is most likely responsible for increased interferon sensitivity of VeroAV, and suggest the importance of $\sigma 1$ s in interferon control. In order to further support this idea, the initiation codon for the

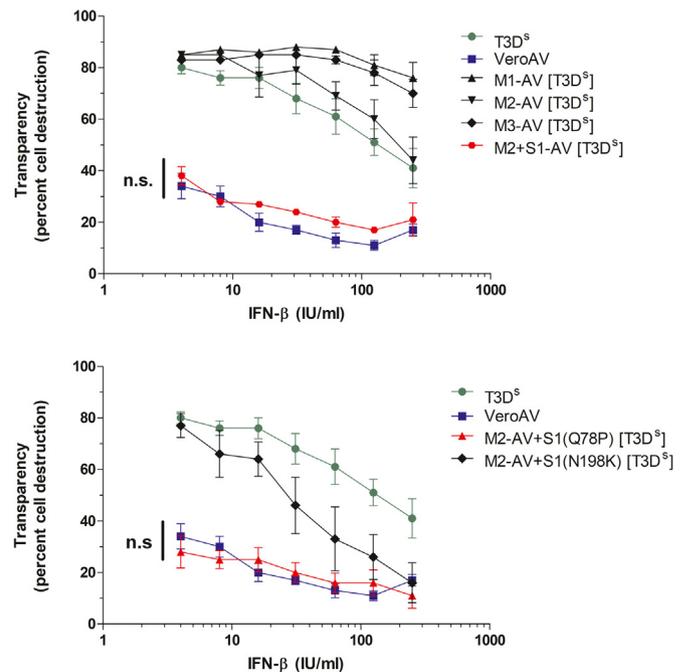


Fig. 3. Sensitivity of reassortant viruses to different interferon concentrations. The same viruses as in Fig. 2 were examined by the interferon dilution assay, as described in Materials and methods. The percentage of cell destruction, as determined by optical density of methylene blue stain compared to mock-infected cells, is presented, as described in Materials and methods. On the upper panel the two parental viruses are compared with viruses harboring a single gene reassortment or the M2-S1 combination while the lower panel presents the two single substitution mutants in S1. Result are presented as the average with error bars representing standard error of the mean ($n = 5$ for T3D^S and VeroAV; $n = 2$ for other viruses in the upper panel and $n = 3$ for other viruses in the bottom panel). Using student's *t*-test to compare results at all different interferon concentrations, only two viruses M2 + S1-AV [T3D^S] and M2-AV + S1(Q78P) [T3D^S] were nonsignificantly different from VeroAV ($p > 0.05$ at all concentrations), as indicated (n.s.).

$\sigma 1$ s protein was mutated from ATG to ACG on the plasmid in order to prevent its synthesis. This was done without affecting the $\sigma 1$ -encoding sequence, thus generating a virus harboring $\sigma 1$ from the wild-type T3D^S in the absence of any $\sigma 1$ s expression, as described by others, (Boehme et al., 2009). Although $\sigma 1$ s was shown to be nonessential to viral replication per se (Rodgers et al., 1998), other data indicate that it plays important roles in cell-cycle arrest and apoptosis consecutive to viral infection (Poggioli et al., 2000, 2001; Boehme et al., 2013). Recently, an additional role in the efficient synthesis of viral proteins was also

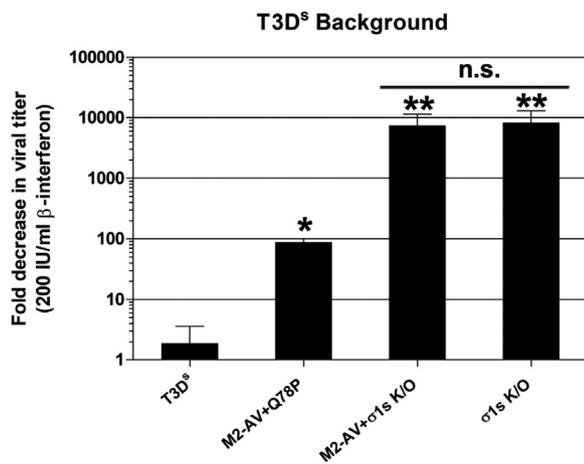


Fig. 4. Interferon sensitivity of the $\sigma 1s$ -null (knockout) virus. Viruses knockout (K/O) for the $\sigma 1s$ protein (in the T3D^S S1 gene) in the presence or absence of the VeroAV M2 gene, as described in the text, were compared with the Q78P mutant virus. Virus titers in the absence or presence of 200 IU/ml of β -interferon were compared, as described in the text. Fold reductions in titers due to interferon presence are presented on a logarithmic scale. Error bars indicate the highest value obtained in at least two replicate experiments. The difference between T3D^S and the mutant was statistically significant at $p < 0.0001$ (*) while the difference between the mutant and either of the two K/O viruses was significant at $p < 0.05$ (**) using Student's *t*-test; there was no significant difference (n.s.) between the two K/O viruses ($p > 0.5$).

observed, at least with some viral strains and cell types (Phillips et al., 2018). In the present study, the T3D^S virus lacking $\sigma 1s$ (T3D^S- $\sigma 1s$ -K/O) was examined for its ability to kill and lyse L929 cells at different multiplicity of infection, as described in previous studies (Lanoie and Lemay, 2018). This revealed a significant decrease in the cytopathic effects of $\sigma 1s$ -K/O virus, especially at low multiplicity of infection (data not shown). This indicates that the $\sigma 1s$ protein does have a more important role in viral replication, and/or effect on the host cell, than initially suspected. This rendered difficult the analysis of interferon sensitivity by the interferon dilution method but the diminution of viral titer could still be used and revealed that this virus is actually a further hundredfold more sensitive to interferon than the virus mutant (Fig. 4). This was also observed either in the presence or absence of the M2 gene of VeroAV, confirming the sole role of the mutation corresponding to Q78P of $\sigma 1$ (N59H in $\sigma 1s$) of VeroAV S1 in interferon sensitivity of the virus mutant and the important role of $\sigma 1s$ as an anti-interferon determinant.

3. Discussion

Efforts are currently underway to further understand reovirus determinants of pathogenesis and oncolytic activity and the interferon response is clearly central in these properties of various strains or isolates. Such efforts should possibly lead to the development of better adapted viruses for anticancer virotherapy applications (Mohamed et al., 2015; Kemp et al., 2016). In previous works, viruses were selected in the laboratory using combinations of chemical mutagenesis, high-passage virus stocks and selection of new viruses. The use of viral persistence in cells exhibiting giving properties could be an interesting approach to select new, more selective, viruses for virotherapy applications (Kim et al., 2007).

Using this viral persistence strategy, the Vero-cell-adapted virus (VeroAV) was previously obtained in the laboratory. This virus exhibits an adaptation at a level of increased binding to sialic acid while also showing increased sensitivity to interferon (Jabre et al., 2013; Sandekian and Lemay, 2015b). This is in accordance with the fact that Vero cells are defective in interferon response. Interestingly, selection

of a virus mutant with increased affinity for sialic acid in murine erythrocytes (tryptophan to basic arginine substitution at position 202) as in the case of VeroAV in Vero cells (asparagine to basic lysine substitution at position 198) previously led to the identification of a $\sigma 1s$ knockout virus (Rodgers et al., 1998). It is tempting to speculate that the gain in infectivity due to increased sialic acid binding somehow compensates for the loss of $\sigma 1s$ in these viruses. Accordingly, the sole introduction of the N59H amino acid substitution and especially the introduction of a stop codon in the $\sigma 1s$ reading frame, was observed to reduce host-cell lysis in the present study. Further work will be needed to determine the impact of $\sigma 1s$ in different viral genetic backgrounds and different cell types.

Induction of and sensitivity to interferon are two interrelated phenomena, as previously shown for example during reovirus infection (Sherry et al., 1998) but they can also be viewed as somewhat independent from each other in some cases. Previous work in the laboratory has shown that a virus selected for increased sensitivity to interferon (Rudd and Lemay, 2005) does not induce increased interferon secretion (Sandekian and Lemay, 2015a). Similarly, this virus does not exhibit apparent differences in the observed changes of cellular gene expression during infection compared to the parental wild-type virus (Boudreault et al., 2016 and unpublished data). More recently, comparisons were also made between T3D^S, the virus laboratory stock, and T3D^K, the virus recovered from the original reverse genetics plasmids (Kobayashi et al., 2007). Increased interferon secretion was attributed to both the $\mu 2$ and $\lambda 1$ protein while increased sensitivity is also partly dependent of $\mu 2$ with $\lambda 2$ also involved (Lanoie and Lemay, 2018).

In previous works, the VeroAV genome was only partly sequenced and it was established that both the $\mu 1$ and $\sigma 1$ proteins are altered (Jabre et al., 2013; Sandekian et al., 2015b). Since $\mu 1$ was altered in a way that affects viral disassembly, it was hypothesized that this could also affect its interaction with its binding partner $\sigma 3$ (Jabre et al., 2013). This protein has long been considered to be important in the control of the interferon-induced protein kinase PKR (Jacobs and Langland, 1998; Samuel, 1998; Schiff, 2008; Sherry, 2009). Although, at the time, this appeared as a logical explanation to explain increased interferon sensitivity exhibited by VeroAV, it turned out not to be the case.

This involvement of $\sigma 1s$ in the interferon sensitivity phenotype was somewhat surprising. The protein was recently shown to be involved in the efficiency of protein synthesis, although not in the same cell type and not the same virus strain (Phillips et al., 2018). It is also known that the protein has at least a partial nuclear localization (Rodgers et al., 1998; Hoyt et al., 2004; Boehme et al., 2011, 2013), suggesting that it may affect the expression of certain genes. Alternatively, the protein might interact with a factor important for the interferon response, beyond induction of interferon per se, as shown for the $\mu 2$ -IRF9 or μ NS-IRF3 interactions (Zurney et al., 2009; Stanifer et al., 2017). The mutant $\sigma 1s$ is still expressed but was found at a reduced level compared to the wild-type by immunoblotting (data not shown). Considering that the interferon sensitivity is at least a hundredfold higher when $\sigma 1s$ is completely absent, even a small reduction in the level of the protein could well be enough to explain the phenotype; however, an additional loss of function cannot be excluded at this point. Clearly the availability of a virus mutant with an intermediate phenotype could facilitate further study of $\sigma 1s$ impact on the interferon response and related effect on viral pathogenesis. Interestingly, approximately 50% of the 120 amino acids of $\sigma 1s$, including asparagine 59, are conserved in 98% or more of the 55 type 3 viral sequences recovered from the NCBI database (data not shown). In contrast, very limited sequence conservations was found between serotypes 1, 2 and 3 (data not shown). It will thus be of interest to compare the effect, if any, of $\sigma 1s$ in other viral serotypes and to identify the common structure involved.

The $\sigma 1s$ protein is well known for its importance in viral propagation in vivo (Boehme et al., 2009, 2011; Nygaard et al., 2013). It is

tempting to speculate that this is due to its role in the control of the interferon response and it will be of interest to pursue these studies. The availability of viruses differing specifically in one viral determinant of interferon induction or sensitivity should certainly contribute to a better understanding of the importance of interferon in viral pathogenesis.

The $\sigma 1s$ protein should thus be added to $\sigma 3$, $\mu 2$, μNS , $\lambda 1$ and $\lambda 2$, as proteins probably involved in either the induction of or sensitivity to the interferon response (Sherry et al., 1998; Sherry, 2009; Sherry et al., 2009; Zurney et al., 2009; Irvin et al., 2012; Stebbing et al., 2014; Sandekian and Lemay, 2015a; Stanifer et al., 2017; Lanoie and Lemay, 2018).

It thus appears that reovirus is one further example of virus coevolution with the innate immune response leading, as previously mentioned, to a loss of the ability of the virus to resist interferon when this selective pressure is removed (Perez-Cidoncha et al., 2014; Hernández-Alonso et al., 2015; Garijo et al., 2016; Weber-Gerlach and Weber, 2016; Du et al., 2018). Interestingly, rotavirus, another dsRNA virus of the Reoviridae family, also uses at least three of its proteins in the control of the interferon response (Sherry, 2009; Sherry et al., 2009; Arnold et al., 2013; López et al., 2015).

This also raises the possibility of combining these determinants in order to further modulate the interferon response. This could lead to further applications of the virus not only as an oncolytic agent but also as a gene or vaccine vector, as proposed previously by various authors (Roner and Joklik, 2001; Rouault and Lemay, 2003; Kobayashi et al., 2007; Brochu-Lafontaine and Lemay, 2012; Demidenko et al., 2013; Mohamed et al., 2015; van den Wollenberg et al., 2015; Boehme et al., 2016; Kemp et al., 2016; Eaton et al., 2017; Stuart et al., 2017).

4. Materials and methods

4.1. Cell lines and viruses

L929 mouse fibroblasts and Vero cells were originally obtained from the American type culture collection (ATCC®). The baby hamster kidney cell line (BHK) stably expressing the T7 RNA polymerase has been described and was a generous gift from the laboratory of Dr. John Hiscott (Lady Davis Research Institute, Montréal, Canada). All cell lines were grown in minimal Eagle medium (MEM) with 5% fetal bovine serum.

Wild-type laboratory stock of reovirus type 3 (T3D^S) was previously described (Lanoie and Lemay, 2018; Sandekian and Lemay, 2015a; 2015b) and recovered by introduction of the appropriate mutations in the plasmids encoding the wild-type virus from Dr. Terry Dermody's laboratory (T3D^K). The Vero-cell-adapted virus (VeroAV) was also previously described (Jabre et al., 2013; Sandekian and Lemay, 2015b). Viruses harboring various combinations of genes from T3D^S and VeroAV were obtained by reverse genetics, as described below. All virus stocks were routinely grown on L929 cells and virus titer determined by TCID₅₀, as described (Danis and Lemay, 1993).

4.2. Sequencing of the whole VeroAV genome

Viral genomic RNA was prepared and fragments for sequencing were generated by RT-PCR, as previously described (Brochu-Lafontaine and Lemay, 2012), except that Vertrel was used in replacement of Freon to prepare reovirus virions (Mendez et al., 2000).

4.3. Reovirus reverse genetics

The plasmids separately harboring each of the cDNA corresponding to the 10 genes of reovirus serotype 3 Dearing, T3D^K, under the transcriptional control of the T7 promoter were originally obtained from the laboratory of Dr. Terence Dermody (Vanderbilt University, Nashville, Tennessee) (Kobayashi et al., 2007). The recovery of the

wild-type laboratory stock T3D^S was initially achieved by first submitting each plasmid to site-directed mutagenesis for each of the gene segment differing between T3D^S and T3D^K (Lanoie and Lemay, 2018; Sandekian and Lemay, 2015a).

The 4 plasmids encoding proteins that differ between the wild-type T3D^S and VeroAV were modified to reconstitute the 4 VeroAV genes; two of them (S1 and M2) were already described (Sandekian and Lemay, 2015b), while the other two (M1 and M3) were obtained by site-directed mutagenesis.

The S1 gene of T3D^S was further modified by site-directed mutagenesis to separately introduce each of the two amino acids substitutions in $\sigma 1$. In another construct, the initiation codon for the small $\sigma 1s$ protein encoded in a second reading frame, was mutated (from ATG to ACG on the plasmid) in order to prevent the synthesis of this second protein, as described by others (Boehme et al., 2009); this construct was referred to as knockout (KO). Sequences of all primers used in mutagenesis are available upon request.

Plasmids were then used to recover infectious virus by the reverse genetics approach using transfection in BHK cells expressing the T7 RNA polymerase (Kobayashi et al., 2010) and rescued viruses were propagated as described before (Brochu-Lafontaine and Lemay, 2012; Sandekian and Lemay, 2015a, 2015b).

4.4. Determination of interferon induction

ELISA assay was performed on different dilutions of the tissue culture medium to determine the concentration of both β -interferon and all subtypes of α -interferon (Verikine mouse interferon beta and alpha ELISA kit, PBL Assay Science).

4.5. Determination of interferon sensitivity

Mouse type I β -interferon was obtained from PBL interferon source. Interferon sensitivity was determined on mouse L929 cells using different approaches. In a first approach, decreased virus titer at near-saturating (200 IU/ml) concentration on L929 cells was measured by TCID₅₀, as previously used (Sandekian and Lemay, 2015a; Lanoie and Lemay, 2018). Secondly, twofold dilutions of interferon were prepared from 250 IU/ml to approximately 4 IU/ml in single wells of a 96 wells microplates seeded with L929 cells; cells were then infected with the different viruses at a MOI of 0.05, incubated for 4–5 days before being fixed and stained with methylene blue, essentially as described (Sandekian and Lemay, 2015a; Lanoie and Lemay, 2018).

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