Identification of Attenuating Mutations on the Reovirus Type 3 S1 Double-Stranded RNA Segment with a Rapid Sequencing Technique

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Reovirus type 3 variants with mutations in the major neutralization domain of the $\sigma 1$ protein have attenuated neurovirulence and restricted neurotropism. We devised a variation of the rapid RNA sequencing technique to facilitate the analysis of double-stranded RNA. We sequenced the S1 double-stranded RNA segment, which encodes the $\sigma 1$ protein, of five attenuated reovirus type 3 variants. Four of the variants have changes in codon 419, and a fifth variant has a change at codon 340, all of which resulted in amino acid substitutions in the $\sigma 1$ protein. We identified two sites on the reovirus type 3 $\sigma 1$ protein that play a critical role in neurovirulence.

We used the mammalian reoviruses as a model to better understand viral pathogenesis and to define at a molecular level the determinants responsible for the capacity of these viruses to infect specific cells and tissues (tropism). The reovirus genome consists of 10 segments of double-stranded (ds) RNA (reviewed in reference 8). Infection of neonatal mice with mammalian reovirus type 3 produces a fatal encephalitis secondary to localization of reovirus type 3 in neurons (10, 13). The reovirus S1 ds-RNA segment encoding the $\sigma 1$ protein (viral hemagglutinin) determines the tropism of reovirus for neurons (21, 23, 24) and pituitary cells (12). The $\sigma 1$ protein is also responsible for determining a number of virus-host cell interactions, including serotype-specific humoral and cellular immunity, binding to cellular microtubules, and inhibition of cellular DNA synthesis (8). To identify different domains of the reovirus type 3 hemagglutinin, we isolated $\sigma 1$ -specific monoclonal antibodies (3). Analysis of the $\sigma 1$ protein by using these monoclonal antibodies indicated that there are at least three distinct domains (20). One domain, termed the neutralization domain, is primarily defined by two monoclonal antibodies, A2 and G5, that show high titers of neutralizing activity. The G5 monoclonal antibody was used to generate viral variants (designated A, F, and K) that resist neutralization (19). These variant viruses are markedly less virulent than the parental reovirus type 3 virus from which they were selected. In addition, examination of brain sections of neonatal mice injected intracerebrally with the variants shows that they cause tissue injury in restricted regions of the mouse brain (18). These results indicate that mutations in the reovirus type 3 $\sigma 1$ protein cause marked alterations in viral virulence and tropism. In addition to the original attenuated reovirus type 3 variants, two more reovirus type 3 viruses with antigenically altered $\sigma 1$ proteins were selected as previously described (19), (designated AI4 and AI7), except that the parental reovirus type 3 was grown in the presence of neutralizing monoclonal antibody A2 instead of G5. Thus,

we have a total of five variants that were selected by resistance to neutralization with two distinct monoclonal antibodies directed against the $\sigma 1$ protein.

In this report, we describe a method that allowed us to identify the attenuating mutation site(s) of the five reovirus variants. We sequenced the entire S1 ds-RNA segment of each of the variants and compared its sequence to that of parental reovirus type 3, which was cloned and sequenced previously (2, 4, 11). To expedite the sequencing of the variant S1 ds-RNA segments, we developed a variation of the RNA sequencing method of G. M. Air (1) to sequence directly from ds RNA by using synthetic primers. This approach eliminated cloning the viral genes and allowed rapid direct sequencing of the S1 genes of five immune-selected reovirus type 3 variants.

MATERIALS AND METHODS

Cells. Mouse L cells were grown in suspension and monolayers in Joklik modified Eagle minimal essential medium (Irvine Scientific) supplemented with 5% fetal calf serum and 2 mM glutamine.

Viruses. Reovirus type 3 (strain Dearing) is the laboratory stock. Variant viruses A, F, and K are immune-selected viruses which have been previously described (19). Variant viruses AI4 and AI7 were immune selected similarly to variants A, F, and K, except that A2 neutralizing monoclonal antibody (3) was used. Purified virus was obtained by growing viral stocks in suspension cultures of L cells at 34°C and extracted from infected cells as previously described (14). Neutralization assays and hemagglutination inhibition assays were essentially done as previously described (3).

Virulence. Virus stocks were grown on L cells as previously described (14), and viral titers were expressed in PFU. Virulence was measured by the dose of virus (PFU) which killed 50% of the mice upon intracerebral injection (LD₅₀). Intracerebral injections (10 μ l) were done on neonatal NIH Swiss mice with various dilutions of virus stock, and animals were observed for 3 weeks for mortality. The LD₅₀ was calculated by the method of Reed and Muench (15).

Purification of ds RNA. Purified virus (2 mg) was incubated in 1% sodium dodecyl sulfate for 15 min and then extracted with phenol-chloroform (1:1). The ds RNA was then ethanol precipitated. To further isolate a particular ds RNA segment,

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TABLE 1. Final nucleotide concentrations for sequencing ds RNA by using synthetic primer and [³⁵S]dATP

Reaction terminator	Nucleotide concn (μM)						
	dGTP	dTTP	dCTP	ddGTP	ddATP	ddTTP	ddCTP
ddGTP	11.1	55.5	55.5	2.8			
ddATP	55.5	55.5	55.5		1.4		
ddTTP	55.5	11.1	55.5			2.8	
ddCTP	55.5	55.5	11.1				2.8

the genomic RNA was subjected to electrophoresis in a 5% polyacrylamide gel, stained with ethidium bromide, excised from the gel, electroeluted, and ethanol precipitated.

Sequencing ds RNA with primers. Synthetic primers were purchased from Clayton Naeve at St. Jude Children's Research Hospital, Memphis, Tenn., and further purified by high-pressure liquid chromatography. RNase inhibitor was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The reverse transcriptase of avian myeloblastosis virus was purchased from Life Sciences, St. Petersburg, Florida. ³⁵S-labeled dATP (600 Ci/mmol) was purchased from Amersham Corp., London, England. The reverse transcriptase buffer used was 0.05 M Tris hydrochloride (pH 8.3)–0.01 M MgCl₂–0.07 M KCl–0.03 M β-mercaptoethanol. The formamide-dye mix used was 0.02 M EDTA–0.03% bromophenol blue–0.03% xylene cyanol in 100% deionized formamide.

The ds RNA was denatured by incubating purified ds RNA (3 μg/μl) in a solution containing 90% dimethyl sulfoxide and 1 mM Tris hydrochloride (pH 6.8) at 50°C for 45 min. The RNA was then placed on ice, diluted 1:5 in deionized H₂O, and mixed with an equal volume of primer (1 unit of optical density [at 260 nm] per ml). All primers used for the sequencing method were 15 nucleotide bases in length. One microliter of RNA-primer mix was immediately diluted into tubes containing deoxynucleotides, dideoxynucleotides, reverse transcriptase buffer, 7 μCi of ³⁵S-labeled dATP (600 uCi/mmol), 3 U of RNase inhibitor, and 5 U of reverse transcriptase in a final volume of 5 μl. The final nucleotide concentrations for each reaction are shown in Table 1. Each reaction mixture was incubated at 37°C for 30 min and then chased with 1 μl of a 1 mM deoxynucleotide solution at 37°C for 10 min. Finally, 4 μl of formamide-dye mix was added, and the sample was boiled for 3 min and loaded onto an 8% sequencing gel (16). By this method it was possible to sequence 250 to 300 nucleotide bases.

RESULTS

Characterization of reovirus newly isolated variants AI4 and AI7. Variants AI4 and AI7 were isolated from parental reovirus type 3 stocks by immune selection in the presence of neutralizing monoclonal antibody A2. Variants AI4 and AI7 showed 90 and 100% resistance, respectively, to neutralization by a 1:500 dilution of monoclonal antibody A2 (Table 2). In comparison, reovirus type 3 showed 10% resistance to neutralization by monoclonal antibody A2 at the same dilution. In addition, variants AI4 and AI7 showed 90 and 80% resistance, respectively, to neutralization by a 1:500 dilution of monoclonal antibody G5. Reovirus type 3 showed 6% resistance to neutralization by monoclonal antibody G5 at the same dilution.

Neurovirulence of variants AI4 and AI7. To determine whether variants AI4 and AI7 exhibited attenuated neurovirulence (as was the case for variants A, F, and K

[Table 3]), we injected the variants at various dilutions intracerebrally into neonatal mice. Variant AI4 showed an LD₅₀ of 8 × 10⁶ PFU, and variant AI7 showed an LD₅₀ of 4 × 10⁶ PFU, indicating that both variants are at least 100,000-fold less virulent than reovirus type 3 (strain Dearing).

Brain sections of mice injected with reovirus type 3 show extensive damage in the cortical and hippocampal regions (18). Upon examination of brain sections of mice injected with variant AI4 or AI7, cell destruction was observed mainly in the hippocampal region, and cortical injury was not seen (data not shown). The pattern of pathological injury was similar to that previously described for variants A, F, and K (18).

Nucleotide mutations on the S1 gene of reovirus type 3 variants. The entire S1 gene, consisting of 1,416 nucleotide bases, was sequenced for all five immune-selected variants. A single nucleotide base substitution was found in each variant (Fig. 1; Table 3). Variants F, K, AI4, and AI7 all showed base substitutions in codon 419 that alter amino acid 419 of the σ1 protein. Variant A had no change at codon 419 but had one nucleotide base substitution at codon 340. This results in a change in the σ1 protein at amino acid 340 (Fig. 1; Table 3). The specific amino acid substitutions associated with these attenuated variants are glutamic acid replaced by lysine (Glu→Lys), Glu→Ala, Glu→Gly, and Asp→Val. These amino acid changes have the potential to significantly alter both the structure and function of the protein.

DISCUSSION

In this report, we have described the sequence of the entire S1 gene of five reovirus type 3 variants by using a rapid sequencing technique. We have identified two sites (amino acid 340 and 419 of the σ1 protein) which are critical for affecting neurovirulence and neurotropism. Previously, three immune-selected variants of reovirus type 3 with markedly decreased neurovirulence and restricted neurotropism were described (19). In this report, we isolated and characterized two additional variants (AI4 and AI7) which were immune selected by using a different neutralizing monoclonal antibody (A2) from that used to select the original variants. We demonstrated that the new variants, AI4 and AI7, like the original variants, have both attenuated neurovirulence and restricted neurotropism. To sequence the series of reovirus variants, we developed a variation of the direct RNA-sequencing method (1) to rapidly sequence ds RNA and used this method to sequence the entire S1 ds RNA segment from each of the five variants. To identify the mutations responsible for altering neurovirulence and restricting neurotropism of these variants, we compared their S1 ds RNA nucleotide sequence to the S1 ds RNA nucleotide sequence of parental reovirus type 3.

The nucleotide base sequence of the S1 ds RNA segment of parental reovirus type 3 predicted that the σ1 protein contains 455 amino acid residues. Analysis of the sequence of the σ1 protein predicts that the secondary structure of the

TABLE 2. Neutralization of reovirus type 3 variants

Virus	Monoclonal antibody (dilution 1:500)	% Resistant
Reovirus type 3	G5	6
Reovirus type 3	A2	10
Variant AI4	G5	90
Variant AI4	A2	90
Variant AI7	G5	80
Variant AI7	A2	100

TABLE 3. Location of attenuating mutations on the reovirus type 3 S1 ds RNA

Virus	Antibody	LD ₅₀ (PFU)	Codon 419	Amino acid 419	Codon 340	Amino acid 340
Reovirus type 3	None	10	GAG	Glutamic acid	GAC	Aspartic acid
Variant A	G5	10 ⁵	GAG	Glutamic acid	GTC	Valine
Variant F	G5	>10 ⁷	AAG	Lysine	GAC	Aspartic acid
Variant K	G5	>10 ⁷	AAG	Lysine	GAC	Aspartic acid
Variant AI4	A2	10 ^{6.9}	GCG	Alanine	GAC	Aspartic acid
Variant AI7	A2	10 ^{6.6}	GGG	Glycine	GAC	Aspartic acid

amino-terminal region of the $\sigma 1$ protein is an α -helical structure enabling multimers of the $\sigma 1$ protein to assume a coiled-coil structure within the virion. Sequence analysis of the carboxy-terminal region of the $\sigma 1$ protein suggests that it has a globular secondary structure. On the basis of the secondary structure data of the $\sigma 1$ protein we proposed a model (2) whereby the $\sigma 1$ protein is anchored to the virion by the α -helical region in the amino-terminal region and the carboxy-terminal region of the $\sigma 1$ protein is exposed on the surface of the virions such that it can interact with host cells. The altered amino acid residue of four attenuated variants, F, K, AI4, and AI7, is at amino acid 419, and the fifth variant, A, is altered at amino acid 340. This result indicates that the sites of mutation are in the carboxy-terminal and not

the α -helical portion of the $\sigma 1$ protein. Thus, the alteration of the variant viruses correlates with the protein regions that are putatively exposed on the viral surface and suggests that the mutation sites might be positioned on the surface of the virion in close proximity to or at the virus-host cell binding site.

In addition to sequencing the entire S1 ds RNA segment of all five variants and locating a point mutation in each S1 ds RNA segment, we prepared viral reassortants containing that S1 gene of variant K and have verified that the S1 gene product of one of the variants (variant K) is solely responsible for the altered phenotype of the variants (9).

Variant A (one of the original variants), a variant that displays an intermediate pattern in terms of virulence (LD₅₀

FIG. 1. Nucleotide sequence analysis of the S1 gene from reovirus type 3 and the viral variants. Shown are gels of ddGTP-, ddATP-, ddTTP-, and ddCTP-terminated reactions (lanes G, A, T, and C, respectively) of reverse transcriptase. Arrowheads indicate the position of the mutation. (A) The sequences shown are in the region of codon 419 of the S1 gene of reovirus type 3 and variants K, F, AI4, and AI7. (B) The sequences shown are in the region of codon 340 of the S1 gene of reovirus type 3 and variant A.

of $<10^5$) and shows an antigenic reactivity of 48% resistance to monoclonal antibody G5, also has an altered amino acid in the carboxy region of the $\sigma 1$ protein. The biological alterations of variant A (attenuation and resistance to neutralization) are not as dramatic as those of the other four variants. This might indicate that the altered amino acid, amino acid 340, is situated in a less critical position in terms of structural conformation of the antigenic site.

Thus, in this report, we have described the sequence changes in five antigenic variants of reovirus type 3 that are attenuated for neurovirulence and restricted in cell tropism. Sequence alterations have recently been determined in antigenic variants, natural isolates, and attenuated strains (vaccines) of a number of viruses, including influenza, poliovirus and rabies (5–7, 17, 22). From these studies, it is clear that a single or limited number of amino acid changes may have profound effects on virus-host interactions, although the precise details of how virulence is altered is not well understood.

The development of rapid sequencing of nucleic acids has made it more practical to analyze larger numbers of viral variants. For single-stranded RNA viruses such as influenza, polio, rabies, and rhinovirus, a rapid sequencing method has been available for some time (1). However, there has been no method in which synthetic primers were used to rapidly sequence ds RNA. The method described in this report will now permit other investigators to sequence ds RNA molecules more rapidly (including other ds RNA viruses). ds RNA viruses have the advantage of having two RNA strands to sequence. Therefore, if there are ambiguities in the sequence of one RNA strand, primers can be synthesized to the complementary strand and the complementary RNA strand can also be sequenced. This makes it possible to confirm the sequence directly.

In conclusion, by studying attenuated reovirus variants that were selected by growth in the presence of neutralizing monoclonal antibodies directed against the reovirus type 3 $\sigma 1$ protein, we have established that amino acids 419 and 340 of the reovirus $\sigma 1$ protein are critical sites that affect reovirus neurovirulence. A new sequencing method has made it possible to quickly sequence that entire S1 ds RNA segment from all the viral variants and to rapidly identify mutation sites.

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