Role of the $\mu 1$ Protein in Reovirus Stability and Capacity To Cause Chromium Release from Host Cells

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The reovirus M2 gene is associated with the capacity of type 3 strain Abney (T3A) intermediate subviral particles (ISVPs) to permeabilize cell membranes as measured by chromium (^{51}Cr) release (P. Lucia-Jandris, J. W. Hooper, and B. N. Fields, *J. Virol.* 67:5339–5345, 1993). In addition, reovirus mutants with lesions in the M2 gene can be selected by heating virus at 37°C for 20 min in 33% ethanol (D. R. Wessner and B. N. Fields, *J. Virol.* 67:2442–2447, 1993). In this report we investigated the mechanism by which the reovirus M2 gene product (the $\mu 1$ protein) influences the capacity of reovirus ISVPs to permeabilize membranes, using ethanol-selected T3A mutants. Each of three T3A ethanol-resistant mutants isolated (JH2, JH3, and JH4) exhibited a decreased capacity to cause ^{51}Cr release relative to that of wild-type T3A. Sequence analysis of the M2 genes of wild-type T3A and the T3A mutants indicated that each mutant possesses a single amino acid substitution in a central region of the 708-amino-acid $\mu 1$ protein: JH2 (residue 466, Tyr to Cys), JH3 (residue 459, Lys to Glu), and JH4 (residue 497 Pro to Ser). Assays performed with reovirus natural isolates, reassortants, and a set of previously characterized type 3 strain Dearing (T3D) ethanol-resistant mutants revealed a strong correlation between ethanol sensitivity and the capacity to cause ^{51}Cr release. We found that ISVPs generated from the T3A and T3D mutants were stable when heated to 50°C, whereas wild-type T3A ISVPs are inactivated under these conditions. Together, these data suggest that amino acid substitutions in a central region of the $\mu 1$ protein affect the capacity of the ISVP to permeabilize L-cell membranes by altering the stability of the virus particle.

Reovirus is a nonenveloped icosahedral virus possessing a segmented genome consisting of 10 double-stranded RNAs (reviewed in reference 28). Three structural forms of reovirus have been identified: the virion, the intermediate subviral particle (ISVP), and the core. ISVPs are generated *in vitro* by treatment with proteases such as chymotrypsin (5, 10), in the gut by treatment with intestinal proteases (4), or in the endosome by treatment with endosomal proteases (30). ISVPs are characterized by the removal of the outer capsid protein $\sigma 3$ (29), by cleavage of the outer capsid protein $\mu 1\text{C}$ to fragments δ (29) and ϕ (26), and by extension of the viral hemagglutinin-cell attachment protein $\sigma 1$ (15). Under certain facilitating conditions, the ISVP form of the virus is further processed, resulting in generation of transcriptionally active core particles that lack the outer capsid proteins ($\sigma 3$, $\sigma 1$, and $\mu 1$ derivatives) and are essentially noninfectious (20, 29).

To date, only two reovirus genes, S1 and M2, have been associated with functions related to the entry of reovirus into mouse L cells. The S1 gene encodes $\sigma 1$, a 455-amino-acid protein whose structure likely forms a fibrous tail and globular head (2, 14, 15). $\sigma 1$ oligomers are located at the 12 icosahedral vertices of the viral particle and function as the virus hemagglutinin and cell attachment protein (1, 15, 21, 32).

The M2 gene encodes $\mu 1$ (23), a 708-amino-acid outer capsid protein that is myristoylated at its N terminus (19, 27). Six hundred copies of $\mu 1$ (a cleaved form of $\mu 1\text{C}$, $\mu 1\text{C}$ is the predominant form in virions) (19, 27) form a lattice that covers the virus core and consist of hexameric arrays of trimeric clusters (13).

Hexameric arrays are interrupted (two of the six trimeric clusters are missing from the interrupted hexamer) where the $\lambda 2$ core spikes protrude through the $\mu 1$ capsid. The M2 gene has been associated with the capacity of reovirus ISVPs generated from whole virus particles or genome-free top component particles to cause membrane damage as determined by an L-cell chromium (^{51}Cr) release assay (22) and a hemolysis assay (24).

Type 3 strain Abney (T3A) reovirus ISVPs, but not intact virions or cores, cause the release of ^{51}Cr when added to preloaded L cells, whereas type 1 strain Lang (T1L) ISVPs do not cause ^{51}Cr release under the conditions of our assay (7, 22). In the present study we have investigated the molecular genetic basis for the M2 gene-linked ^{51}Cr release polymorphisms observed among different reoviruses. Our data indicate that mutations in a central region of the $\mu 1$ protein affect the capacity of the ISVP to permeabilize L-cell membranes by altering the stability (as determined by resistance to denaturing conditions) of the virus particle or domains of the particle. In addition, we present evidence that the intact $\sigma 1$ protein is required for ^{51}Cr release, suggesting that ISVP $\sigma 1$ -mediated attachment to L cells is required for ^{51}Cr release.

MATERIALS AND METHODS

Cells and viruses. The cells used in all experiments were spinner-adapted murine L929 cells (L cells) obtained from laboratory stocks. Cells were maintained as previously described (22).

The reovirus natural isolates used in this study, T3A, T1L, type 3 strain Dearing (T3D), and type 2 strain Jones (T2J), were obtained from laboratory stocks. Reassortants EB144 and GW524 were obtained from laboratory stocks and have been described previously (9, 22). Purified virus was isolated by freeze extraction and isopycnic centrifugation through CsCl as previously described (15). The virion concentration was determined by measuring A_{260} , assuming that 1 A_{260} unit is 2.1×10^{12} particles (29). Plaque assays were performed as previously described (15).

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Preparation of ISVPs. ISVPs were prepared as previously described (22). Purified virions were diluted to the indicated concentration in virion storage buffer (150 mM NaCl, 10 mM MgCl₂, 10 mM Tris [pH 7.5]) containing 200 µg of chymotrypsin (type VII *N*-α-*p*-tosyl-L-lysine chloromethylketone [TLCK]-treated bovine α-chymotrypsin [Sigma Chemical Co., St. Louis, Mo.]) per ml and placed at 37°C for 20 min. Protease digestion times were varied as indicated. Digestion was terminated by adding phenylmethylsulfonyl fluoride to 2 mM, and the mixture was stored at 4°C until used. The ISVP concentration was determined by measuring *A*₂₆₀, assuming that 1 *A*₂₆₀ unit is 2.72×10^{12} ISVP particles (11).

To confirm successful ISVP generation (and to determine if the σ1 protein was intact after chymotrypsin digestion), ISVPs were routinely subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE) followed by silver staining of the protein.

Chromium release assay. The ⁵¹Cr release assay used in this study has been described previously (22). Briefly, ISVPs were generated by digestion with chymotrypsin, and the indicated concentration of particles was added (100 µl per well) to prechilled L-cell monolayers (48-well plates [Costar, Cambridge, Mass.] containing $\sim 4 \times 10^5$ cells) preloaded with ⁵¹Cr. After 1 h of adsorption, warm medium (37°C) was added to each well, and the plate was placed at room temperature for 150 min, at which time the amount of ⁵¹Cr released into the medium was determined as previously described (22).

Ethanol sensitivity assay. Viruses (second or third passage stocks) were diluted in buffered gel saline (gel-saline, 10 mM HEPES [N-hydroxyethylpiperazine-*N'*-ethanesulfonic acid] [pH 7.4]) (GSH) containing either no ethanol or a final concentration of 33% ethanol (Aaper, Shelbyville, Ky.) and incubated for 20 min in a 37°C water bath. Inactivation was terminated by diluting samples 1:10 in cold GSH. Infectivity remaining after treatment was determined by plaque assay (15). The titers of samples treated with no ethanol or 33% ethanol were compared, and the fold reduction in titer was determined. This method is identical to the method described by Wessner and Fields (34), except 10 mM HEPES was added to the gel-saline as a buffer.

Cloning and sequencing M2 gene. The T3A M2 gene was partially sequenced by a rapid RNA sequencing method described by Basel-Duby et al. (3). RNA sequence information was used to design primers for cloning the M2 gene of wild-type T3A and mutants JH4, JH3, JH2, F212A.2, and GW524 by reverse transcription (RT)-PCR. The M2 gene was reverse transcribed with a primer designated AS-2 and Moloney murine leukemia virus RT (GIBCO-BRL, Gaithersburg, Md.). The AS-2 primer (5'-CGCGCGGCCGATCGATGATTTGCCTGCATCCCTTAA-3') is 36-mer complementary to 23 nucleotides at the 3' end of the plus strand of the T3A M2 gene and includes an additional 13 nucleotides (5' end of primer) that supply an *EagI* restriction site. RT products were PCR amplified with Vent polymerase (New England Biolabs, Beverly, Mass.) as follows (30 cycles): 30 s at 94°C, 1 min at 60°C, and 72°C for 2.5 min. Primer AS-2 and a second primer AS-1 were used in the PCRs. The AS-1 primer (5'-GGCGGGCCCAAGATGGGGAACGCTTCC-3') is 30-mer complementary to nucleotides 25 to 44 at the 3' end of the minus strand of the T3A M2 gene and includes an additional 10 nucleotides (5' end of primer) that supply an *ApaI* restriction site. PCR products and plasmid pGEM-5zf(–) (Promega Corp., Madison, Wis.) were digested with *EagI* and *ApaI*, and both insert and vector were purified by electrophoresis through low-gelling-temperature agarose (FMC Bio-products, Rockland, Maine) followed by phenol extraction. M2 inserts were ligated into pGEM-5zf(–) with T4 DNA ligase (New England Biolabs). Recombinant plasmids were introduced into competent JM109 cells (Promega) by electroporation, and recombinant plasmids containing the M2 gene were selected by standard methods. M2 DNA was amplified, purified by polyethylene glycol 8000 (Sigma) precipitation, and sequenced by the dideoxy method with T7 polymerase (Sequenase version 2.0; United States Biochemical, Cleveland, Ohio) and appropriate primers. This cloning strategy omitted the first 24 nucleotides at the 5' end of the M2 gene (noncoding region). All mutations were confirmed by sequencing DNA purified from clones obtained from independent PCR amplifications.

Nucleotide sequence accession number. The T3A M2 gene has been submitted to GenBank and EMBL under accession no. U24260. The sequences of the M2 gene from T2J (35) and T3D (19) have been published previously. The sequence of the M2 gene of the Field's laboratory T1L (used in these experiments) differs from the published T1L M2 sequence (35) at four nucleotide positions; two of the four differences result in amino acid changes (Leu to Pro at 272 and Arg to Lys at 284) (18).

RESULTS

⁵¹Cr release phenotypes of prototypic reovirus strains and comparison of M2 gene products. T3A ISVPs, but not intact virions, cause ⁵¹Cr release from mouse L cells (7). T1L ISVPs failed to cause ⁵¹Cr release under the conditions of our assay, and this strain difference maps to the M2 gene (22). To determine the molecular basis for this T3A versus T1L polymorphism, we cloned and sequenced the M2 gene of T3A and compared it with the T1L M2 sequence (18). At the nucleic

FIG. 1. Comparison of μ1 protein predicted amino acid sequences of reovirus prototypic strains. The T3A μ1 protein amino acid sequence deduced from the nucleic acid sequence is shown in single-letter code. The deduced T3D (19), T1L (18), and T2J (35) amino acid sequences were aligned relative to the T3A sequence. Changes in the amino acid sequence are indicated; identical residues are represented by dashed lines. ♦, changes unique to strains that fail to cause ⁵¹Cr release (Fig. 2); ▼, endogenous cleavage site at residue 42 and exogenous chymotrypsin site at residue 581 (19); ▽, trypsin stable fragment of μ1 located between residues 284 and 506 (26); *, myristoylated glycine at position 2 (27).

acid level, the T3A M2 gene differs from the T1L M2 gene at 311 of the 2,203 bases (data not shown). At the protein level, T3A μ1 differs from T1L μ1 at 13 amino acid residues (Fig. 1). Nucleotide differences were confirmed by cloning and sequencing an independent T3A clone. Because the difference in ⁵¹Cr release phenotypes between T3A and T1L is associated with the M2 gene, one or more of the 13 amino acid differences must modify the μ1 protein so as to alter the ability of the ISVP to interact with and/or permeabilize L-cell membranes.

We tested additional reovirus prototypic strains with known M2 sequences for their capacity to cause ⁵¹Cr release. T3D and T2J reoviruses were digested to ISVPs, and a range of particle concentrations were tested for their effects on ⁵¹Cr release. T3A and T1L ISVPs were included as controls. ISVPs of T3A and T3D caused significant levels of ⁵¹Cr release, whereas ISVPs of T1L and T2J did not cause significant levels of ⁵¹Cr release at ISVP concentrations as high as 2.5×10^{12} particles per ml (6.25×10^5 particles per cell) (Fig. 2). The decreased level of ⁵¹Cr release observed for T3D in Fig. 2 may reflect the partial removal of σ1 after 20 min of chymotrypsin digestion (see below).

Comparison of the deduced amino acid sequences of the M2 gene products of T3A, T3D, T1L, and T2J revealed that the μ1 protein is highly conserved, with variations among these five isolates occurring at only 28 of the 708 amino acid residues (Fig. 1). T3A differs from T3D at 4 residues, from T1L at 13 residues, and from T2J at 20 residues (Fig. 1). Variation in the

FIG. 2. ^{51}Cr release by reovirus prototypic strains. Viruses (3.0×10^{12} particles (p) per ml) were digested to ISVPs, diluted in virion storage buffer (0.33×10^{12} , 0.75×10^{12} , 1.5×10^{12} , 1.8×10^{12} , and 2.5×10^{12} particles per ml), and tested in the ^{51}Cr release assay as described in Materials and Methods. ^{51}Cr release was determined after 150 min at room temperature. The percentage of ^{51}Cr release was determined relative to the level of ^{51}Cr released when 4% Triton X-100 was added in place of ISVPs. Each point represents the mean value for two independent experiments $\pm$ the standard error of the mean.

M2 gene product appears to occur mainly in four regions of the protein: at the N terminus of $\mu 1\text{C}$ (51 to 128), between residues 305 and 459, between residues 471 and 519, and near the C terminus (659 to 693) (Fig. 1). Twenty-four amino acid positions contain amino acid changes that are unique to strains that do not cause ^{51}Cr release (T1L and T2J), and six of the changes are shared by T1L and T2J. Because multiple changes were present in all natural isolates tested, we were unable to discern which change(s) was responsible for altering the capacity of the virus (ISVP) to cause ^{51}Cr release.

ISVP attachment to L cells is required for ^{51}Cr release. We suspected that the decrease in ^{51}Cr release by T3D ISVPs shown in Fig. 2 might be due to the protease-sensitive nature of the T3D $\sigma 1$ protein (20, 25). To test this, T3A and T3D were digested with chymotrypsin for increasing lengths of time and then tested for ^{51}Cr release. Extended chymotrypsin digestion times did not affect the capacity of T3A to cause ^{51}Cr release. However, T3D ^{51}Cr release was reduced at 20 min and not detected after 40 min of digestion (Fig. 3A). SDS-PAGE analysis indicated that particles gained the capacity to cause ^{51}Cr release when $\mu 1\text{C}$ was cleaved to δ and $\sigma 3$ was degraded (first time point was 10 min). At later times, the T3D $\sigma 1$ protein was cleaved and this loss of intact $\sigma 1$ correlated with a decrease in the capacity to cause ^{51}Cr release (Fig. 3B). In contrast, the T3A $\sigma 1$ protein was resistant to chymotrypsin digestion, and T3A ISVPs did not lose the capacity to cause ^{51}Cr release after extended chymotrypsin digestion.

In a separate study we have observed that a monoclonal antibody specific to $\sigma 1$ (MAb-G5) inhibits ^{51}Cr release by blocking ISVP attachment to L cells (16). Together, these findings indicate that ISVP attachment to L cells via $\sigma 1$ is a prerequisite for ISVP-induced ^{51}Cr release. In the experiments for which the results are shown in Fig. 2 and in all other ^{51}Cr release experiments, ISVP samples were subjected to SDS-PAGE and silver stained to confirm digestion of $\mu 1\text{C}$ to δ ,

B.

FIG. 3. T3D loss in capacity to cause ^{51}Cr release correlates with loss of $\sigma 1$ protein. T3D and T3A at 1.5×10^{12} particles per ml were digested with 200 μl of chymotrypsin per ml at 37°C for the indicated lengths of time. Reactions were terminated with phenylmethylsulfonyl fluoride, and the resulting particles were either tested for the capacity to cause ^{51}Cr release or subjected to SDS-PAGE. Time zero samples received virion storage buffer in place of chymotrypsin. (A) Samples were tested for the capacity to cause ^{51}Cr release as described in Materials and Methods. Release was determined after 150 min at room temperature. The percent release was determined relative to the level of ^{51}Cr release in wells in which 4% Triton X-100 was added in place of ISVPs. Each point represents the mean $\pm$ standard deviation for two parallel determinations. (B) Samples were boiled 2 min in sample buffer (125 mM Tris [pH 8.0], 0.01% bromophenol blue, 10% glycerol, 1% SDS, 2% 2-mercaptoethanol), loaded (3.0×10^{10} particles per lane) onto an SDS-10% PAGE minigel, and subjected to 20 mA for ~ 2 h. Proteins were visualized by silver staining according to the manufacturer (Bio-Rad). Viral proteins and protein fragments are labeled on the left, and the positions of marker proteins are labeled on the right (M_r s in thousands). F, dye front; CHT, chymotrypsin. Digestion times (in minutes) are indicated above the lanes.

degradation of $\sigma 3$, and the integrity of the $\sigma 1$ protein (data not shown).

Generation of T3A ethanol-resistant mutants. Given that the $\mu 1$ proteins of the prototypic strains of reovirus contained multiple, scattered positions of amino acid variability, it was impossible to determine which region of the protein was re-

FIG. 4. Ethanol sensitivity of T3A and T3A ethanol-resistant mutants. (A) T3A was tested for ethanol sensitivity by incubating a P2 stock at 37°C in GSH (pH 7.4) containing no ethanol (ETOH) or 33% ethanol for the indicated lengths of time. A plaque assay was used to quantify the number of PFU remaining after treatment. (B) Three T3A ethanol-resistant mutants selected by the method of Wessner and Fields (34) were tested for ethanol sensitivity as described in Materials and Methods. Wild-type T3A and wild-type T1L were included as controls. The fold reduction in the number of PFU remaining after ethanol treatment is indicated. Each bar represents the mean for at least two independent experiments $\pm$ the standard error of the mean.

sponsible for the ⁵¹Cr release polymorphism. An indirect method was needed to select T3A viruses with mutations, preferably single amino acid substitutions, in the μ 1 protein. Reovirus sensitivity to inactivation by ethanol maps to the M2 gene (12). Our laboratory had previously demonstrated that 33% ethanol could be used to select T3D ethanol-resistant mutants containing lesions in the M2 gene (34). This method was used to generate T3A mutants.

To determine if T3A is sensitive to ethanol (and therefore amenable to mutant selection), second passage T3A was incubated in 33% ethanol in GSH (pH 7.4) for 0, 10, 20, 40, and 80 min at 37°C and infectious virus was quantitated by a plaque assay. The titer of T3A was reduced greater than 4 logs within 10 min of 33% ethanol treatment at 37°C (Fig. 4A). However, a fraction of the treated virus remained infectious after extended ethanol treatment. The 4 log₁₀ reduction in titer, and the existence of an ethanol-resistant population, suggested that it should be possible to select T3A ethanol-resistant mutants.

T3A-infected cell lysates (second passage stocks [P2]) derived from independent plaque picks were treated with 33% ethanol in GSH (pH 7.4) for 20 min at 37°C. Plaque assays were performed, and putative ethanol-resistant mutants were picked, twice plaque-purified, and amplified by passing twice through tissue cultures (P2 stocks). P2 stocks from plaque-purified viruses were retested for ethanol resistance. Mutant viruses, isolated from three separate T3A clones, were designated JH2, JH3, and JH4. Mutant JH4, followed by JH3 and JH2, exhibited the greatest ethanol resistance (Fig. 4B). All

FIG. 5. ⁵¹Cr release by T3A mutants. Three T3A ethanol-resistant mutants (JH2, JH3, and JH4) were digested to ISVPs and tested for the capacity to cause ⁵¹Cr release as described in the legend to Fig. 2. Each point represents the mean for two independent experiments $\pm$ the standard error of the mean. Values for controls T3A and T1L are identical to those in Fig. 2.

three mutants were at least 100 times more resistant to ethanol than wild-type T3A. Each mutant exhibited a different plaque size: JH4 plaques were smaller than T3A plaques, JH3 plaques were larger than T3A plaques, and JH2 plaques were essentially the same size as T3A plaques (data not shown).

T3A ethanol-resistant mutants exhibit ⁵¹Cr release-negative phenotypes. The T3A ethanol-resistant mutants were amplified, purified, digested to ISVPs, and tested for the capacity to cause ⁵¹Cr release as described in Materials and Methods. Surprisingly, all three T3A ethanol-resistant mutants caused decreased levels of ⁵¹Cr release relative to that of wild-type T3A (Fig. 5). Each mutant caused less than 15% ⁵¹Cr release, even at concentrations as high as 2.5×10^{12} particles per ml. Wild-type T3A ISVPs caused approximately 60% ⁵¹Cr release at concentrations of $\geq 1.5 \times 10^{12}$ particles per ml. T1L ISVPs caused less than 5% release. This result indicated that we had successfully isolated mutant T3A viruses with ⁵¹Cr release-negative phenotypes by using ethanol selection.

⁵¹Cr release-negative phenotype of JH4 maps to M2 gene. After T3A mutants with ⁵¹Cr release-negative phenotypes were generated, a backcross reassortant virus experiment was performed to determine the gene(s) with which the mutant phenotype segregated. A representative T3A mutant, JH4, was crossed with reovirus EB144 to generate reassortant virus progeny containing combinations of gene segments from each parent. EB144 is a reassortant virus possessing seven T1L genes and three T3D genes including the M2 gene and two nonstructural genes, S3 and M3 (9). EB144 was used in this reassortant analysis for two reasons. First, EB144 possesses the capacity to cause a significant level of ⁵¹Cr release (Table 1). Second, all JH4 gene segments encoding structural proteins can be distinguished from the corresponding EB144 genes by SDS-PAGE electrophoretotype analysis (data not shown).

From mixed infections of JH4 and EB144, we obtained a panel of eight reassortant viruses containing various combinations of JH4 and EB144 gene segments. The viruses were plaque purified at least two times, amplified, purified, digested

TABLE 1. Backcross of T3A ^{51}Cr release-negative mutant JH4 with virus EB144

Virus ^a	Genotypic mobility of JH4 × EB144 reassortants ^b										Specific ⁵¹ Cr release ^d (% total ± SEM [n])	Ethanol sensitivity ^e (log ₁₀ reduction in titer ± SEM [n])
	Outer capsid				Core				Nonvirion ^c			
	S1	S4	M2	L2	S2	M1	L1	L3	S3	M3		
JH4	J	J	J	J	J	J	J	J	J	J	9 ± 2 (5)	1.1 ± 0.4 (3)
EB144	E	E	E	E	E	E	E	E	E	E	38 ± 4 (5)	4.6 ± 0.1 (4)
F175A	J	E	J	E	J	J	E	E			10 ± 1 (2)	2.3 ± 0.1 (2)
F64A.2	E	J	J	J	J	J	J	J			1 ± 1 (2)	2.8 ± 0.6 (3)
F64B.2	E	E	J	J	J	J	J	J			1 (2)	2.5 ± 0.5 (3)
F204A.1	J	E	J	E	J	J	J	E			3 (3)	3.3 ± 0.1 (7)
F52A.1	E	E	E	J	E	E	E	J			55 ± 3 (2)	5.2 (2)
F44A.2	J	J	E	J	J	J	E	J			44 ± 7 (2)	5.3 ± 0.1 (2)
F37A.2	E	E	E	E	E	J	E	E			45 ± 4 (2)	5.1 ± 0.2 (2)
F212A.2	E	J	J ^f	E	J	J	E	J			18 ± 1 (3)	4.9 ± 0.1 (2)

^a Purified virions at 1.8×10^{12} particles per ml were digested to ISVPs and were diluted to 1.5×10^{12} particles per ml for use in ^{51}Cr release assays. Infected cell lysate (P3) stocks were used for the ethanol sensitivity assays.

^b J, the genome segment runs with a JH4 mobility; E, the genome runs with EB144 mobility. The number of exceptions to the segregation were three, four, zero, and four for outer capsid protein-encoding gene segments S1, S4, M2, and L2, respectively, and one, two, one, and four for core protein-encoding gene segments, S2, M1, L1, and L3, respectively.

^c The JH4 and EB144 nonvirion protein encoding gene segments were not distinguishable by electrophoretotyping analysis.

^d Measured after 150 min (postadsorption) at room temperature. Each experiment consisted of two or more parallel determinations.

^e The ethanol sensitivity assay was performed as described in Materials and Methods.

^f The M2 gene segment runs with an altered mobility on SDS-PAGE.

to ISVPs, and tested for the capacity to cause ^{51}Cr release. The results of the reassortant analysis are presented in Table 1. Reassortants that caused low levels of ^{51}Cr release (10% or less ^{51}Cr release) contained an M2 gene from JH4. Reassortants that caused high levels of ^{51}Cr release (greater than or equal to the ^{51}Cr release caused by EB144, 38%) contained an M2 gene from EB144. One reassortant (F212A.2) caused an intermediate level of ^{51}Cr release (18%) and was the subject of further study (see below). Thus, the JH4 $\times$ EB144 backcross reassortant analysis indicated that the decreased capacity of JH4 ISVPs to cause ^{51}Cr release segregates with the JH4 M2 gene segment. There was at least one exception to segregation for all other structural gene segments.

Ethanol resistance or sensitivity of the reassortants was also assessed. JH4, EB144, and the reassortants were tested for resistance to 33% ethanol as described in Materials and Methods. Although all of the reassortants were more ethanol sensitive than the parent JH4, the reassortants could be divided into two groups on the basis of their ethanol sensitivity (Table 1). One group was $\leq 2.5 \log_{10}$ times more sensitive to ethanol than JH4. These viruses possessed the M2 gene segment from JH4. The second group was $\geq 3.5 \log_{10}$ times more sensitive than JH4. These viruses possessed the M2 gene segment from EB144. The same virus that caused an intermediate level of ^{51}Cr release (reassortant F212A.2) apparently contained an M2 gene from JH4 but was ethanol sensitive (4.9 $\log_{10}$ reduction in titer). This virus was the subject of further study (see below). Thus, the ethanol-resistant phenotype of mutant JH4 mapped to the M2 gene.

Although the reassortants containing a JH4 M2 gene were more resistant to ethanol than viruses containing an EB144 M2 gene, none of the reassortants was as resistant as the parent virus JH4. We suspect that the difference in ethanol sensitivities between JH4 and the stable reassortants (F175A, F64A.2, F64B.2, and F204A.1) reflects an intrinsic instability of particles assembled from proteins derived from different strains of the virus (i.e., proteins that have not coevolved). The results of the reassortant virus analysis presented in Table 1 coupled with

the results of the analysis by Wessner and Fields (34) and the original ethanol sensitivity mapping performed by Drayna and Fields (12) clearly demonstrate that the M2 gene plays a dominant role in determining the stability of reovirus when treated with 33% ethanol at 37°C.

Sequences of M2 genes of T3A ethanol-resistant mutants.

To determine if the T3A mutants with ^{51}Cr release-negative phenotypes have lesions in the M2 genes as predicted, we cloned and sequenced the M2 genes from JH4, JH3, and JH2 by RT-PCR. Sequence analysis revealed that each virus had a different single base change in the M2 gene. The double-stranded RNA of the JH4 M2 gene contains a cytidine-to-uridine change at nucleotide 1518 of the plus strand; the JH3 M2 gene contains an adenosine-to-guanosine change at nucleotide 1404; and the JH2 M2 gene contains an adenosine-to-guanosine change at nucleotide 1426. Each base change results in a distinct missense mutation in the μ 1 protein. The amino acid substitution for each mutant is shown in Table 2 and represented schematically in Fig. 6). This sequence information coupled with the backcross analysis of JH4 suggests that a single amino acid substitution in the JH4 μ 1 protein (Pro to Ser at 497) is responsible for both the ethanol resistance of the virus and the decreased capacity of the virus (ISVP) to cause ^{51}Cr release. Backcross experiments were not performed with JH2 or JH3. However, it is likely that the single amino acid substitutions in JH2 and JH3 are responsible for the altered ethanol and ^{51}Cr release phenotypes of those viruses. In support of this premise, a previously characterized T3D ethanol-resistant mutant designated 3a9 contains an amino acid substitution (Lys to Gln) at the same position (amino acid position 459) as the JH3 substitution (34). Wessner and Fields had previously performed a backcross analysis with mutant 3a9 to prove that ethanol resistance maps to the M2 gene (34).

Reassortant F212A.2 contains an intragenic suppressor mutation. Reassortant virus F212A.2 caused an intermediate level of ^{51}Cr release (18%) and was ethanol sensitive (4.9 $\log_{10}$ reduction in titer) (Table 1). This phenotype for an intermediate level of ^{51}Cr release was confirmed by using indepen-

TABLE 2. Correlation between capacity to cause ^{51}Cr release and ethanol sensitivity

Virus	Origin of M2	Amino acid substitution ^a	Reference ^b	^{51}Cr release ^c	Ethanol sensitivity ^d
T3A	T3A	None (wild type)		+	+
JH4	T3A	Pro-497-Ser		—	—
JH3	T3A	Lys-459-Glu		—	—
JH2	T3A	Tyr-466-Cys		—	—
F212A.2	JH4	Ser-496-Cys		±	+
T3D	T3D	None (wild type)	19	+	+
3a5	T3D	Ala-319-Glu	34	—	—
3d5	T3D	Val-425-Phe	34	—	—
3b4	T3D	Gln-440-Lys	34	—	—
3a3	T3D	Pro-454-Arg	34	—	—
3a9	T3D	Lys-459-Gln	34	—	—
T1L	T1L	None (wild type)	18	—	—
GW524	T1L	Ala-439-Ser		±	+
T2J	T2J	None (wild type)	35	—	—

^a The amino acid of the wild-type virus, the position, and the amino acid of mutant virus (from left to right) are given.

^b Reference for sequence data if other than this report.

^c The ^{51}Cr release assays were performed as described in Materials and Methods. ISVPs at 1.5×10^{12} particles per ml were tested for the capacity to cause ^{51}Cr release (150 min at room temperature). +, ^{51}Cr release greater than 30%; —, ^{51}Cr release less than 15%; ±, ^{51}Cr release between 15% and 30%.

^d The ethanol sensitivity assay was performed as described in Materials and Methods. Infected cell lysates of passaged viruses (usually P3 stocks) were tested. +, ethanol treatment resulted in a more than 10,000-fold reduction in PFU titer; —, less than 10,000-fold reduction in titer.

dently passaged virus (data not shown). Initial electrophoretic analysis of F212A.2 indicated that the M2 gene appeared to be derived from JH4. However, on careful inspection of the electropherotype, we observed that the M2 gene segment from F212A.2 ran slightly faster than the M2 gene segment from JH4 (data not shown). This mobility difference coupled with the intermediate level of ^{51}Cr release and ethanol sensitivity suggested that the M2 gene of this virus may contain a mutation that suppresses the Pro-to-Ser substitution at residue 497 of the JH4 $\mu 1$ protein, resulting in an intermediate level of ^{51}Cr release and ethanol sensitivity.

To determine if F212A.2 contains a second site mutation in

$\mu 1$, the M2 gene was cloned and sequenced by RT-PCR. Sequence analysis indicated that the M2 gene of F212A.2 was derived from JH4. However, in addition to the original Ser-to-Pro substitution at position 497, F212A.2 contained a second nucleotide change (nucleotide 1516, cytidine to guanosine) that resulted in a Ser-to-Cys substitution at position 496 (Table 2). Interestingly, this amino acid substitution is located at a position adjacent to the original mutation at 497. Thus, a single amino acid substitution adjacent to the original mutation can apparently suppress two seemingly unrelated mutant phenotypes, ethanol resistance and decreased ability to cause ^{51}Cr release.

T3A $\times$ T1L reassortant GW524 contains a mutation in M2. In the study in which the ^{51}Cr release phenotype was mapped to the M2 gene (22), one reassortant, designated GW524, exhibited an intermediate level of ^{51}Cr release. GW524 is a T3A $\times$ T1L reassortant virus containing a M2 gene from T1L (by electropherotyping). Interestingly, the $\mu 1$ protein of this virus runs with an altered mobility (more slowly) (22) when subjected to SDS-PAGE, suggesting that the altered ^{51}Cr release phenotype was caused by a mutation in the M2 gene of GW524.

We cloned and sequenced the M2 gene of GW524 by RT-PCR and found that the gene was identical to the T1L M2 gene except for a single nucleotide change (uridine to guanosine at nucleotide 1344). This base change results in a missense mutation (Ser to Ala) at amino acid position 439 (Table 2). As in the T3A and T3D mutants, the mutation in GW524 $\mu 1$ is located within the central region of the protein (Fig. 6).

When tested for resistance to 33% ethanol at 37°C for 20 min, the titer of GW524 was reduced 4.9 logs, indicating that the virus is ethanol sensitive. When tested in the ^{51}Cr release assay, GW524 ISVPs caused intermediate levels of ^{51}Cr release (20%), confirming previous results (22). These results (summarized in Table 2) provide additional evidence that the central region of $\mu 1$ is important for modulating both ethanol sensitivity and the ability to cause ^{51}Cr release. Moreover, these results indicate that a mutation in the central region of the $\mu 1$ protein can increase the capacity to cause ^{51}Cr release.

Correlation of ethanol sensitivity and capacity to cause ^{51}Cr release. Our ethanol sensitivity and ^{51}Cr release results from the viruses thus far tested (T3A, T3D, T1L, the T3A mutants [JH4, JH3, and JH2], EB144 $\times$ JH4 reassortants, pseudorevertant F212A.2, and GW524) led us to hypothesize that the ethanol resistance phenotype and ^{51}Cr release-negative phenotype are directly related. Viruses (ISVPs) that caused ^{51}Cr release were ethanol sensitive, and viruses that failed to cause ^{51}Cr release were ethanol resistant. T2J was relatively ethanol resistant (2.1 $\log_{10}$ reduction in titer), supporting our hypothesis.

To further investigate the correlation between ethanol sensitivity and the capacity to cause ^{51}Cr release, we tested a number of previously characterized T3D ethanol-resistant mutants (34) for their ability to cause ^{51}Cr release. Five T3D ethanol mutants, each with a defined mutation in the $\mu 1$ protein (34), were digested to ISVPs (15-min chymotrypsin digestion was used to avoid cleavage of the $\sigma 1$ protein [Fig. 3]) and tested for the capacity to cause ^{51}Cr release as described in Materials and Methods. Each T3D ethanol-resistant mutant (3a5, 3d5, 3b4, 3a3, and 3a9) caused less than 12% ^{51}Cr release, whereas wild-type T3D ISVPs caused greater than 50% release in these experiments (Table 2).

The ethanol resistance or sensitivity and ^{51}Cr release phenotypes of the viruses used in this study are summarized in Table 2. These results indicated that ethanol sensitivity and the capacity to cause ^{51}Cr release are correlated: viruses that were

FIG. 6. Mutations in a central region of $\mu 1$ affect the capacity of reovirus ISVPs to cause ^{51}Cr release. T3A mutants (□), T3D mutants (○), reassortant F212A.2 (mutant) (■), and reassortant GW524 (mutant) (▲) each contain single amino acid substitutions that cluster in a central region of the $\mu 1$ protein (Table 2). The protein is myristoylated at amino acid position 2 (*) (27). The predominant form of $\mu 1$ in virions is $\mu 1\text{N}/\mu 1\text{C}$, which is generated when $\mu 1$ is cleaved between positions 42 and 43 by an endogenous protease (19). When ISVPs are generated with chymotrypsin, $\mu 1\text{C}$ is cleaved between positions 581 and 582 to generate fragments δ and ϕ (19, 26). Cores generated with trypsin retain a trypsin-stable fragment (TSF) of $\mu 1$ spanning positions 285 to 506 (24). Predicted amphipathic helices are located at positions 534 to 551 and positions 591 to 604 (α^*) (26).

FIG. 7. T3A ethanol-resistant mutants and T3D ethanol-resistant mutants are thermostable relative to wild-type T3A and T3D. Wild-type viruses T3A, T1L, and T3D and ethanol-resistant viruses JH2, JH3, JH4, 3a5, 3d5, 3b4, 3a3, and 3a9 were tested for ability to retain infectivity when incubated at 50°C for increasing lengths of time. ISVPs were generated at 1.5×10^{12} particles per ml in a 50- μ l volume and then diluted 1:10 in gel-saline-10 mM HEPES (pH 7.4). Samples were divided into four 50- μ l aliquots. One aliquot was placed on ice (time zero), and the other three samples were held at 50°C for the indicated lengths of time and then placed on ice. Infectivity remaining after heat treatment was determined by plaque assay. Points represent the means for two to four separate experiments $\pm$ standard deviations.

sensitive to 33% ethanol (20 min at 37°C) caused ^{51}Cr release, and viruses that were resistant to 33% ethanol (20 min at 37°C) exhibited a diminished capacity to cause ^{51}Cr release.

Thermostability of ethanol-resistant mutants. Viruses that were not capable of causing significant levels of ISVP-induced ^{51}Cr release showed ethanol resistance (Table 2), which we interpreted as increased particle stability. To gain additional evidence for this interpretation, we tested a number of viruses, including the T3A and T3D ethanol-resistant mutants, for sensitivity to heat. ISVPs were prepared as described in Materials and Methods and placed in a 50°C water bath for 0, 1, 5, and 10 min. After heat treatment, infectivity remaining in the sample was quantitated by plaque assay. The titer of wild-type T3A and wild-type T3D was reduced $\sim 3 \log_{10}$ after 10 min at 50°C. In contrast, the T3A mutants (JH2, JH3, and JH4) and T3D mutants (3a5, 3d5, 3b4, 3a3, and 3a9) retained their infectivity (titer reduction was less than $1 \log_{10}$), as did T1L (Fig. 7). Thus, ISVPs of the T3A and T3D mutants were relatively resistant to thermal denaturation. ISVPs were used in these experiments to avoid involvement of the $\sigma 3$ protein, which is an important determinant in reovirus resistance or sensitivity to heat (12). These results provide further evidence that the capacity of reovirus ISVPs to permeabilize membranes is related to particle stability.

DISCUSSION

The principal goal of this study was to gain insights into the mechanism by which reoviruses enter host cells. We have investigated the molecular genetic basis for a previously described M2 gene-linked polymorphism in the capacity of reovirus ISVPs to induce ^{51}Cr release from L cells (22). Our results indicate that a central region of the $\mu 1$ protein can play a role in dictating whether reovirus ISVPs will cause ^{51}Cr release

under the conditions of our assay. Moreover, we have found that there is a correlation between the facility by which reovirus ISVPs cause ^{51}Cr release and the resistance of reovirus to inactivation by denaturing conditions: viruses that are sensitive to denaturing conditions (20 min at 37°C in 33% ethanol or 10 min at 50°C) are capable of causing ISVP-induced ^{51}Cr release, whereas viruses resistant to denaturing conditions are deficient in causing ^{51}Cr release.

$\sigma 1$ -mediated attachment is a prerequisite for ^{51}Cr release.

Before discussing the role of $\mu 1$ in membrane interaction and penetration, it is important to note that there are at least three lines of evidence that ^{51}Cr release by reovirus ISVPs requires $\sigma 1$ -mediated attachment of ISVPs to L cells. First, this study and previous studies (8, 22) have demonstrated that the level of ^{51}Cr release increases as the ISVP concentration increases and then reaches a plateau, suggesting the ISVP receptor sites on the L-cell surface are being saturated. Second, we have found that a neutralizing monoclonal antibody specific to $\sigma 1$ (MAb-G5) inhibits ^{51}Cr release by interfering with ISVP attachment to L cells (16). Finally, in this study we have determined that extended chymotrypsin digestion of T3D during ISVP generation correlates with a loss of the capacity to cause ^{51}Cr release and that this loss corresponds with proteolytic digestion of the cell-attachment $\sigma 1$ protein. It should be noted that $\sigma 1$ -mediated attachment is not required for other assays used to study membrane disruption by reovirus ISVPs, including ISVP-induced channel formation in lipid bilayers (31) and hemolysis (24). Thus, the ^{51}Cr release assay used in these experiments determines ISVP attachment as well as ISVP-induced membrane permeabilization.

Sequence comparison of natural isolates. Initial experiments using prototypic reovirus strains indicated that although the M2 gene product ($\mu 1$) is highly conserved among the four strains studied (variation at only 28 of the 708 amino acid positions), the specific amino acid change(s) responsible for affecting the ^{51}Cr release phenotype could not be ascertained. These amino acid changes do not occur at known cleavage sites or in motifs postulated to play a role in membrane interaction. Interestingly, 10 of the 28 variable positions are located in the C-terminal ϕ fragment region of $\mu 1$. Nine of the 10 changes are unique to the two strains of reovirus that do not cause ^{51}Cr release (T1L and T2J). Nibert and Fields have previously suggested that the ϕ fragment of $\mu 1$, because of its high net positive charge, may be involved in membrane interaction (e.g., via electrostatic interactions with a negatively charged cell membrane) (26). The changes in T1L and T2J ϕ relative to the T3A and T3D ϕ do not involve charged residues. Therefore, if the change(s) in ϕ is responsible for the ^{51}Cr release polymorphism, then it alters a property of the protein other than its charge. In addition to the 10 changes in ϕ , variable positions are also found clustered in regions of the δ fragment, including 10 variable positions in the central region of $\mu 1$. The $\mu 1\text{N}$ fragment amino acid sequence is completely conserved for T3A, T1L, T3D, and T2J, indicating that this region is not responsible for the differences in the ^{51}Cr release phenotypes.

Mutations in a central region of $\mu 1$ affect both virus stability and capacity of ISVPs to cause ^{51}Cr release from L cells.

We used an M2 gene-specific selection technique (33% ethanol for 20 min at 37°C) previously described (34) to isolate three T3A ethanol-resistant mutant viruses (JH2, JH3, and JH4) that were also ^{51}Cr release-negative mutants. The ^{51}Cr release-negative phenotype of one representative mutant (JH4) was mapped to the M2 gene segment by backcross reassortant virus analysis. Sequence analysis of the M2 gene segment of the T3A mutants indicated that each mutant contained a different single amino acid substitution in a central

region of the $\mu 1$ protein. We tested other viruses, including five previously characterized T3D ethanol-resistant mutants (34), for the capacity to cause ^{51}Cr release and ethanol sensitivity and found that ethanol resistance and the capacity to cause ^{51}Cr release correlated. We also tested these viruses for thermostability and found that the viruses that readily caused ISVP-induced ^{51}Cr release (T3A and T3D) were heat sensitive whereas the viruses that failed to cause ^{51}Cr release (the T3A and T3D ethanol-resistant mutants and T1L) were not inactivated when heated at 50°C . Together these results suggest that the capacity of reovirus ISVPs to permeabilize membranes is related to the stability of the virus and that the stability of the virus is influenced by a central region of the $\mu 1$ protein.

The JH4 $\times$ EB144 backcross yielded one reassortant (F212A.2) that possesses a JH4 M2 gene with a putative intragenic suppressor mutation. Interestingly, the mutation (Ser to Cys at position 496) involves the amino acid adjacent to the JH4 mutation at position 497. Both the ethanol-resistant phenotype and the ^{51}Cr release phenotype of this virus were altered, presumably by the second site mutation. We also found that a mutation in the T1L M2 (reassortant GW524, Ala to Ser at position 439) could increase the capacity of the virus to cause ^{51}Cr release and increase the sensitivity of the virus to ethanol. Together these results confirmed the finding that a central region of $\mu 1$ is important for stability (as measured by 20 min at 37°C in 33% ethanol) (34) and demonstrated for the first time that this central region of $\mu 1$ plays a role in dictating the facility by which reovirus interacts with and permeabilizes membranes as determined by ^{51}Cr release from L cells.

The T3A ethanol-resistant mutants, T3D ethanol-resistant mutants, pseudorevertant F212A.2, and mutant GW524 all contain lesions in a central region of $\mu 1$ protein previously proposed to play a role in virus stability (34). A schematic diagram of the $\mu 1$ protein and the positions of the mutations listed in Table 2 is presented in Fig. 6. Natural isolates that fail to cause ^{51}Cr release, T1L and T2J, contain lesions within the region of $\mu 1$ defined by the ethanol-resistant mutants (T1L, three changes; T2J, eight changes; two changes are common to both viruses [Fig. 1]). It seems likely that one or more of the changes in the central region of the T1L and T2J $\mu 1$ are responsible for the difference in ^{51}Cr release phenotypes; however, the possibility that amino acid substitutions in other regions of the protein influence the ^{51}Cr release phenotype of these natural isolates cannot be ruled out.

The region of $\mu 1$ containing the mutations that affect the ^{51}Cr release phenotype does not contain features previously proposed to play a role in reovirus interaction with target cell membranes (a myristoylated glycine at $\mu 1$ position 2 [27], two amphipathic α helices on either side of the δ - ϕ cleavage site, and a highly basic C-terminal fragment, ϕ [26]). The mutations lie within a fragment of $\mu 1$ (between amino acid residues 285 and 506) that remains protease resistant when cores are generated with trypsin (24). The trypsin-stable fragment of $\mu 1$ contains an epitope(s) recognized by monoclonal antibodies that bind to intact ISVPs (17). This indicates that at least part of the domain of $\mu 1$ that contains the ^{51}Cr release-ethanol mutants (the trypsin stable fragment) is exposed on the ISVP surface where it can interact with the external environment (i.e., bind antibodies). Interestingly, two monoclonal antibodies that recognize the $\mu 1$ trypsin-stable fragment inhibit ^{51}Cr release when bound bivalently to ISVPs (16).

Role of $\mu 1$ protein in particle stability and membrane permeabilization. We propose that the central region of $\mu 1$, defined by the ethanol-resistant mutants, regulates ISVP conformational change and that this conformational change facilitates membrane interaction and permeabilization. A confor-

mational change in the ISVP could result in the exposure of regions of the particle that insert into and disrupt membranes allowing part or all of the particle access to the cytoplasm.

Under certain *in vitro* conditions, ISVPs undergo a dramatic conformational change and convert to core particles (core conversion) which are transcriptionally active (5, 8, 20, 29). If chymotrypsin is present during or after the conformational change, the $\mu 1$ outer capsid layer is degraded because of exposure of previously masked protease sites. If protease is absent during ISVP conversion, then the particles flocculate, probably because of hydrophobic interactions. It is possible that a similar, or identical, ISVP conformational change is involved in membrane penetration by reovirus cores or core-like particles.

One condition that triggers core conversion *in vitro* is high particle concentration (particle concentration-facilitated core conversion) (6). Interestingly, some viruses convert to cores at lower particle concentrations than other viruses. For instance, T3A and T3D require lower particle concentrations than does T1L to convert to cores. This polymorphism in core conversion appears to correlate with a polymorphism in the capacity to cause ^{51}Cr release. T3A and T3D convert to cores at relatively low particle concentrations and cause ^{51}Cr release, whereas T1L requires a high particle concentration to convert to cores and does not cause ^{51}Cr release. Moreover, Wessner observed that T3D ethanol-resistant mutants (which we determined are ^{51}Cr release mutants) required higher particle concentrations than did wild-type T3D to convert to cores (33). The observation that the capacity of particles to convert to cores and the capacity to cause ^{51}Cr release appear to correlate suggests that these phenomena may be related. This finding is consistent with the idea that the central region of $\mu 1$ regulates particle stability, particle stability dictates ISVP conformational change, and ISVP conformational change facilitates membrane disruption and particle penetration.

Conclusions. In summary, we have found that single amino acid substitutions in a central region of the $\mu 1$ protein affect the capacity of reovirus ISVPs to cause ^{51}Cr release. In addition, we have determined that the capacity of reovirus ISVPs to cause ^{51}Cr release correlates with virus sensitivity to denaturing conditions (33% ethanol, 37°C , 20 min; or 50°C , 10 min), suggesting that the stability of $\mu 1$ tertiary or particle quaternary structure influences the capacity to cause ^{51}Cr release. This correlation coupled with the observation that viruses that readily cause ^{51}Cr release convert to cores at lower concentrations than viruses that do not cause ^{51}Cr release suggests that membrane permeabilization by reovirus ISVPs is directly related to the phenomenon of core conversion. We speculate that the region of $\mu 1$ defined by the ^{51}Cr release and ethanol-resistant mutants is involved in regulating core conversion. This regulation may be based on the relative strengths of intraparticle interactions that stabilize the ISVP or the capacity of exposed regions of $\mu 1$ to interact with external signals that destabilize the ISVP and trigger membrane permeabilization, or both.

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