Reovirus M2 Gene Is Associated with Chromium Release from Mouse L Cells

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In this study, we investigated the interaction of reovirus particles with cell membranes by using a ^{51}Cr release assay. We confirmed prior observations (J. Borsa, B. D. Morash, M. D. Sargent, T. P. Copps, P. A. Lievaart, and J. G. Szekely, *J. Gen. Virol.* 45:161–170, 1979) that intermediate subviral particles (ISVPs) of reovirus type 3 strain Abney (T3A) induced the release of ^{51}Cr from preloaded L cells and showed that the intact virion and core forms did not. Reovirus type 1 strain Lang (T1L) ISVPs were found to be less efficient at ^{51}Cr release than T3A ISVPs. Reassortants between these strains indicated that the ^{51}Cr release phenotype segregates with the M2 gene segment. Biochemical studies indicated that the ISVPs' acquisition of the capacity to induce ^{51}Cr release followed the cleavage of the viral M2 gene product $\mu 1/\mu 1\text{C}$ to fragments δ and ϕ during virion conversion to ISVP but did not directly correlate with this cleavage. These studies suggest that the reovirus M2 gene product (in its cleaved form) plays a role in interacting with cell membranes.

The interaction of enveloped animal viruses (such as influenza virus) with cell membranes has been extensively studied (19, 29, 30). The viral envelope is known to fuse with the host cell envelope to allow penetration of the membrane by the viral nucleocapsid. Since nonenveloped animal viruses (such as reoviruses) are naked, they must interact with and penetrate the membrane by a different process. Although recent studies with nonenveloped viruses (such as poliovirus, rhinovirus, and reovirus [24, 28]) have provided important insights concerning the nature of viral binding to receptors and entry into cells, the precise manner by which these viruses interact with cellular membranes to deliver their genomes to the cytoplasm is poorly understood.

The mammalian reoviruses are nonenveloped RNA viruses that contain double-stranded segmented genomes (11). Reovirus virions consist of two protein capsid layers (15), with the outer capsid comprised of two major proteins, $\sigma 3$ and $\mu 1/\mu 1\text{C}$, the virus cell attachment protein $\sigma 1$, and the spike protein $\lambda 2$, which extends to the outer capsid from the viral core (12, 17, 27). The inner core of the virus, which contains the viral transcriptase activity (7), consists of the $\lambda 1$, $\lambda 2$, $\lambda 3$, $\mu 2$, and $\sigma 2$ proteins, which enclose 10 segments of viral double-stranded RNA (27).

Reovirus replication occurs in the cytoplasm of infected cells. Receptor-bound virus is taken up into clathrin-coated pits and internalized by receptor mediated endocytosis (26). Within acidified vesicles, the virion is uncoated to an intermediate subviral particle (ISVP), which has lost its outer capsid protein $\sigma 3$ and whose $\mu 1\text{C}$ protein has been proteolytically cleaved to δ and ϕ (1, 3, 14, 20). Further processing of the ISVP results in formation of the core particle, which has lost all of the outer capsid proteins except $\lambda 2$, and is transcriptionally active.

Formation of the ISVP has been shown to be a necessary step in reovirus replication (5, 18, 21, 28). How the ISVP or transcriptionally active core particle gains access to the cytoplasm from a vesicle is unknown. The particle must in

some manner disrupt the integrity of the endosomal/lysosomal membrane in order to allow access to the cytoplasm.

To understand how reovirus interacts with membranes, we investigated the finding that a digested form of reovirus type 3 strain Abney (T3A) is capable of causing the release of ^{51}Cr from preloaded L cells. Initially, we confirmed the observation that T3A ISVPs, but not intact virions, are capable of causing the release of ^{51}Cr from preloaded L cells. We then showed that ISVPs generated from virions lacking RNA (top component) are capable of causing ^{51}Cr release and that core particles of T3A are incapable of causing ^{51}Cr release. Differences in the capacity of type 1 strain Lang (T1L) and T3A ISVPs to induce ^{51}Cr release allowed us to show that the M2 gene product is responsible for this phenotype by use of reassortants. The kinetics by which the M2 gene product undergoes cleavage ($\mu 1\text{C}$ to δ and ϕ) during conversion of virions to ISVPs indicated that the particle's acquisition of the capacity to induce ^{51}Cr release followed the $\mu 1\text{C}$ cleavage, supporting our conclusion that the M2 gene product, in its cleaved form, is a determinant of the interaction of reovirus with membranes.

MATERIALS AND METHODS

Cells and viruses. Mouse L cells were maintained in suspension in Joklik's modified minimal medium (Irvine Scientific, Santa Ana, Calif.) supplemented with 5% fetal bovine serum (HyClone Laboratories Inc., Logan, Utah) and 2 mM glutamine (Irvine Scientific) (cMEM). Reovirus strains T1L, T3A, and type 3 Dearing (T3D) and T3A $\times$ T1L reassortant viruses (viruses possessing different combinations of genes from two different parents) (31) were used in these studies. Virus and top component (virions lacking the RNA component are less dense than full virions and band above virions on CsCl gradients, hence the name "top component") was purified from infected cells as previously described (10) and stored at 4°C in 150 mM NaCl–10 mM MgCl_2 –10 mM Tris-HCl (pH 7.5) (dialysis buffer). Concentration was determined by measuring A_{260} (26). To radioactively label reovirus proteins, the cells were infected as described above, with the addition of 10 μCi of [^{35}S]methio-

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nine (Tran³⁵S-Label; ICN Biomedicals, Inc., Irvine, Calif.) per 2×10^8 cells in 400 μ l at the time of infection.

In vitro chymotrypsin digestion of reovirus to ISVPs or cores. To generate ISVPs or cores, purified reovirus particles were diluted into dialysis buffer, and type VII *N* α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK)-treated α -chymotrypsin (Sigma Chemical Co., St. Louis, Mo.) was added to final concentration of 200 μ g/ml. Digestion mixtures were incubated in a 37°C water bath for 20 to 30 min. Proteolysis was terminated by the addition of phenylmethylsulfonyl fluoride (Sigma) to a concentration of 2 mM, and the ISVPs were stored at 4°C until use. ISVPs were generated by digestion of T3A and T3D at a concentration less than 2×10^{12} particles per ml and of T1L at a concentration of less than 10^{13} particles per ml. At higher concentrations, conversion to core particles occurred.

Chromium release assay. (i) Method A. Forty-eight-well clusters (Costar, Cambridge, Mass.) were seeded with 2×10^5 cells per well and incubated at 37°C in 5% CO₂ for 3 to 6 h. Sodium chromate (Na₂⁵¹CrO₄; New England Nuclear, Boston, Mass.) was added to the wells to yield a final concentration of 10 μ Ci of ⁵¹Cr per ml. The monolayers were incubated overnight at 37°C in 5% CO₂. On the next day, monolayers were washed three times with cMEM either at room temperature or at 4°C. Virions, ISVPs, cores, or top-component ISVPs were adsorbed to the monolayers in a volume of 0.1 ml for 30 min at room temperature or 4°C. Mock infection was achieved by the addition of 0.1 ml of dialysis buffer. Total ⁵¹Cr available for release by the cells was determined by the addition of 0.1 ml of 4% Triton X-100 (Sigma) to the wells. At the end of the adsorption period, monolayers were overlaid with 0.5 ml of cMEM and incubated at 37°C in 5% CO₂. Samples were collected at given time points by removing 0.2-ml aliquots of medium from the wells and placing them into a V-bottom 96-well microtiter plate (Costar). Any cells present in the medium were pelleted by 10 min of centrifugation at 800 rpm in a Sorvall RT6000B tabletop centrifuge (E. I. duPont de Nemours, Wilmington, Del.), and 0.1 ml of supernatant was removed to be assayed for ⁵¹Cr. Samples were usually assayed in duplicate or triplicate, and the average of the replicates was calculated. Samples were counted for 1 min in a gamma counter (Pharmacia LKB model 1272; Pharmacia LKB, Gaithersburg, Md.). Percent total release was determined by dividing the average sample counts per minute by the average total release counts per minutes and multiplying the result by 100.

(ii) Method B. Method B differs slightly from the procedure described above and was used in the genetic study (Fig. 2 and Table 3). L cells were diluted to 4×10^5 cells per ml in cMEM containing 10 μ Ci of Na₂⁵¹CrO₄ per ml and 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES; pH 7.4). Then 0.5 ml of cell suspension was added to the wells of 48-well plates (Costar) and incubated overnight at 37°C in 5% CO₂. The plates were prechilled on ice for 10 min and then washed four times with 0.5 ml of ice-cold MEM containing 10 mM HEPES (pH 7.4) (wash solution) per well. After aspiration of the final wash, 0.1 ml of ISVPs generated at a concentration of 1.8×10^{12} particles/ml and diluted in dialysis buffer to the indicated concentration was added to wells and allowed to adsorb for 1 h on ice. At the end of the adsorption period, 0.4 ml of warm (37°C) cMEM per well was added, and the plates were placed at room temperature (23°C) (plates were sealed with flypaper to prevent medium alkalization). After 150 min, the amount of ⁵¹Cr released into the medium was assessed by transferring

400 μ l from each well into a new 48-well plate. Detached cells were centrifuged as described above, and 300 μ l of cell-free supernatant was counted in a gamma counter. The percentage of specific ⁵¹Cr release was determined by subtracting average background release (control wells to which dialysis buffer was added in place of ISVP) and determining the release relative to average total release (control wells to which 4% Triton X-100 was added in place of ISVPs) as follows: % specific release = [(average sample cpm - average background cpm)/(average total cpm - average background cpm)] $\times$ 100.

SDS-PAGE. Reovirus proteins were separated on sodium dodecyl sulfate (SDS)-10% polyacrylamide gels as described by Laemmli (16). Virus samples were prepared for polyacrylamide gel electrophoresis (PAGE) in sample buffer (125 mM Tris [pH 8.0], 2% 2-mercaptoethanol, 1% SDS, 0.01% bromophenol blue) and boiled for 1 to 2 min. The samples were then loaded into lanes of the gel and subjected to 140 mA $\cdot$ h of electrophoresis. Radioactive gels were soaked for 60 min in fixative (30% isopropanol, 10% acetic acid) and then placed in enlightening solution (duPont) for 15 min. Gels were dried under vacuum and exposed to Kodak Xomat-AR film (Eastman Kodak, Rochester, N.Y.) at -70°C. Nonradioactive gels were soaked in fixative overnight, stained for 10 min (0.5% Coomassie brilliant blue R-250 [Sigma], 30% isopropanol, 10% acetic acid), and then destained (15% isopropanol, 5% acetic acid) for 4 h.

RESULTS

Distinct forms of reovirus differ in the capacity to cause chromium release. A ⁵¹Cr release assay was used to determine which form(s) of reovirus has the capacity to cause membrane perturbations that might result in the leakage of cytoplasmic contents. Previous studies by Borsa et al. have shown that digestion of reovirus T3A to ISVPs in vitro generates a particle which is capable of causing the release of radioactivity from cells which have been incubated with ⁵¹Cr (2). To gain further insight into the particle which is capable of causing ⁵¹Cr release, we tested ISVPs generated from virions and from top component, intact virions, and core particles in the ⁵¹Cr release assay.

When T3A ISVPs (viral particles that have lost their outer capsid protein σ 3 and whose μ 1C protein has been cleaved to δ and ϕ [20]) were tested in the ⁵¹Cr release assay, they caused the rapid release of ⁵¹Cr from preloaded cells, while intact virions resulted in no detectable release of ⁵¹Cr (Table 1, experiment 1). This result shows directly that ISVPs, but not intact virions, are capable of causing ⁵¹Cr release, confirming the data of Borsa et al. (2). We digested T3A under conditions to produce core particles (particles which, in addition to lacking σ 3, lack σ 1 and all fragments of the μ 1 protein) and tested these particles for the capacity to cause ⁵¹Cr release. We found that like intact particles, cores were incapable of causing the release of ⁵¹Cr from preloaded cells (Table 1, experiment 2). A second strain of type 3 reovirus, T3D, was tested in similar experiments. T3D ISVPs, but not virions or cores, caused ⁵¹Cr release; however, T3D ISVP ⁵¹Cr release was highly variable, and usually a lower percentage of total ⁵¹Cr release was observed compared with T3A ISVP ⁵¹Cr release (data not shown).

To determine whether the RNA component of ISVPs is necessary for membrane perturbation, we generated ISVPs from gradient-purified T3A top-component particles and tested them in the ⁵¹Cr release assay. We found that although they are noninfectious, ISVPs generated from reovi-

TABLE 1. ^{51}Cr release caused by different forms of reovirus T3A^a

Form of virus ^b	% Total ^{51}Cr release ^c		
	Expt 1	Expt 2	Expt 3
Mock (dialysis buffer)	1.6 $\pm$ 0.3	2.8 $\pm$ 0.1	4.2 $\pm$ 0.0
Virions	1.3 $\pm$ 0.0	ND	ND
ISVPs	57.6 $\pm$ 4.6	37.6 $\pm$ 0.4	58.0 $\pm$ 1.3
Cores	ND	2.0 $\pm$ 0.0	ND
Top-component ISVPs	ND	ND	58.0 $\pm$ 0.0

^a The ^{51}Cr release assay (method A) was performed as described in Materials and Methods.

^b Purified virions, ISVP digests, and core digests were used at 10^{12} particles per ml.

^c Measured after 60 min in experiment 1 and after 90 min in experiments 2 and 3. Each value represents the mean of two to three replicate wells plus or minus the standard deviation. ND, not determined.

rus T3A top component were as efficient in releasing ^{51}Cr from preloaded cells as were ISVPs that contain a full component of double-stranded RNA (Table 1, experiment 3).

Characterization of chromium release. Having determined that the ISVP is either the particle or the immediate precursor of the particle that causes ^{51}Cr release, we wanted to further characterize the conditions under which ^{51}Cr release occurs. To determine whether cells are induced to release ^{51}Cr by a threshold number of ISVPs or whether leakage is associated with each ISVP particle, various concentrations of T3A ISVPs were adsorbed to preloaded L cells in the ^{51}Cr release assay. As the concentration of ISVPs increased, the amount of ^{51}Cr released increased linearly at low concentrations and eventually reached a plateau ($\approx 70\%$ of total ^{51}Cr loaded), suggesting that some aspect of the system was being saturated (Fig. 1).

To determine whether ^{51}Cr release is an event which requires a fluid membrane or whether it is a detergent-like lysis, we performed the ^{51}Cr release assay at 6°C . When ISVPs were adsorbed at 6°C for 60 min and the subsequent incubation also carried out at 6°C , no release of ^{51}Cr occurred (Table 2). However, if the 6°C adsorption was followed by incubation at 37°C , ^{51}Cr was released from the cells upon elevation of the temperature (Table 2). To be certain that ISVP adsorption took place during the 60-min 6°C

FIG. 1. Relationship of the amount of virus adsorbed versus the level of ^{51}Cr released. The indicated concentrations of T3A were tested in the ^{51}Cr release assay (method A; see Materials and Methods). The amount of ^{51}Cr released at 0, 60, and 120 min postadsorption was measured. Total release was 7,282 cpm.

TABLE 2. Effects of temperature on reovirus T3A ISVP-induced ^{51}Cr release^a

Sample ^b	% Total ^{51}Cr release ^c	
	6°C incubation	37°C incubation
Mock (dialysis buffer)	1.3 $\pm$ 0.0	5.5 $\pm$ 0.4
Virions	ND	4.7 $\pm$ 0.6
ISVPs	1.4 $\pm$ 0.0	46.0 $\pm$ 0.3

^a The ^{51}Cr release assay (method A) was performed as described in Materials and Methods except that adsorption of particles to monolayers was performed on ice.

^b Purified virions and ISVP digests were used at a concentration of 10^{12} particles per ml.

^c Measured after 60 min (postadsorption) at the indicated temperature. Each value represents the mean of two to three replicate wells plus or minus the standard deviation. ND, not determined.

incubation, we removed unadsorbed ISVPs by washing cells extensively after the 6°C adsorption period and found that when the monolayer was warmed to 37°C , ^{51}Cr was efficiently released (data not shown). These results indicate either that a fluid membrane is needed for the ^{51}Cr to be released, that ISVPs require temperatures greater than 6°C to induce membrane permeabilization, or both. In addition, these data demonstrate that ISVP attachment is not sufficient to induce ^{51}Cr release.

Identifying a viral gene which is associated with the ISVPs' capacity to release ^{51}Cr . Strains of reovirus often exhibit polymorphisms with regard to their biological and biochemical properties. Through reassortant analysis, these strain variations can be used to determine the viral gene segments which are associated with a particular phenotype (4, 8). We tested reovirus T1L ISVPs in the ^{51}Cr release assay and found that, unlike T3A and T3D, they did not cause a significant release of ^{51}Cr from preloaded cells under the condition of the assay (Fig. 2).

We used a set of reassortant viruses derived from the

FIG. 2. T1L ISVPs do not induce the release of ^{51}Cr under conditions in which T3A ISVPs do induce ^{51}Cr release. The indicated concentrations of T3L and T3A ISVPs were tested in the ^{51}Cr release assay (method B; see Materials and Methods). ISVPs (0.1 ml per well) were allowed to bind to prechilled monolayers for 1 h on ice, then 0.4 ml of 37°C cMEM was added, and plates were placed at room temperature. ^{51}Cr released into the medium was measured at the indicated times. Percent total specific release was calculated as described in Materials and Methods. Total release was 21,179 cpm. Error bars represent the standard deviation of the mean of duplicate ISVP digests.

TABLE 3. ^{51}Cr release caused by T3A $\times$ T1L reassortants^a

ISVP ^b	Electropherotype of T3A × T1L reassortants ^c										Specific ⁵¹ Cr release (% of total ± SEM [n]) ^d
	Outer capsid				Core				Nonstructural		
	S1	S4	M2	L2	S2	M1	L1	L3	S3	M3	
T3A	A	A	A	A	A	A	A	A	A	A	57 ± 7 (5)
T1L	L	L	L	L	L	L	L	L	L	L	0 ± 1 (5)
GW15	L	L	A	A	L	A	A	L	A	L	64 ± 13 (3)
GW14	L	L	A	L	A	L	L	A	A	A	47 ± 3 (3)
GW20	L	A	A	L	A	A	L	L	L	L	46 ± 7 (3)
GW7	A	A	A	L	L	L	L	L	A	A	45 ± 5 (3)
GW28	A	A	A	L	A	L	L	L	A	L	44 ± 1 (2)
GW27	A	L	L	L	A	A	A	L	L	A	9 ± 4 (3)
GW26	L	L	L	L	L	L	L	A	L	L	6 ± 1 (2)
GW13	A	A	L	A	A	A	A	L	A	A	6 ± 1 (2)
GW12	A	L	L	L	L	A	A	L	L	L	2 ± 0 (2)
GW10	L	L	L	L	A	L	L	A	L	A	2 ± 0 (2)
GW34	L	A	L	L	L	L	L	L	L	L	1 ± 0 (2)
GW11	L	L	L	L	A	L	A	L	A	A	0 ± 0 (2)
GW4	L	A	L	L	L	L	A	L	A	A	−3 ± 0 (2)
Exceptions to segregation	6	5	0	5	6	6	9	6	5	9	
GW524 (intermediate phenotype)	L	A	L ^e	A	A	L	L	A	A	L	21 ± 2 (3)

^a The ^{51}Cr release assay (method B) was performed as described in Materials and Methods.

^b Purified virions at 1.8×10^{12} particles per ml were digested to ISVPs as described for method B and were diluted to 1.5×10^{12} particles per ml for use.

^c A, the genome segment runs with a T1A mobility; L, the genome segment runs with a T1L mobility.

^d Measured after 150 min (postadsorption) at room temperature. Within each experiment, samples were assayed in triplicate wells.

^e The M2 product (μ 1) runs with altered mobility on SDS-PAGE relative to T1L and T3A μ 1 and μ 1 cleavage products μ 1C and δ .

parent strains T3A and T1L (31) to determine which gene(s) was responsible for the ^{51}Cr release phenotype strain difference. ISVPs were prepared from the reassortant viruses and tested in the ^{51}Cr release assay (Table 3). We found that with a single exception, the reassortant ISVPs segregated into two groups: those that caused the release of more than 40% of the total ^{51}Cr from preloaded cells, and those that caused the release of less than 10% of the total ^{51}Cr from preloaded cells. When the reassortants were grouped according to their behavior in this assay, all of the reassortants capable of causing greater than 40% ^{51}Cr release possessed an M2 gene segment from the T3A parent, while those that did not cause significant levels of ^{51}Cr release possessed an M2 gene segment derived from the T1L parent. Thus, the M2 gene segment was linked to differences in the capacity of the different strains (Abney and Lang) to release ^{51}Cr . There were at least five exceptions to segregation for every other gene segment.

One reassortant (GW524; Table 3) possessing a T1L M2 gene segment exhibited an intermediate phenotype (21% specific ^{51}Cr release). Preliminary evidence suggests that GW524 has a mutation in its M2 gene. When GW524 virions were analyzed by SDS-PAGE, the M2 gene product bands μ 1 and μ 1C ran more slowly than the same bands from either T1L or T3A (Fig. 3). When GW524 ISVPs were analyzed by SDS-PAGE, the δ fragment of μ 1 ran with altered mobility (data not shown). This finding indicates that the δ region of the M2 gene product, which spans amino acids 43 to 581 of the M2 product (13, 20), contains a mutation. It seems likely that the mutation in the M2 gene of GW524 is responsible for the intermediate ^{51}Cr release phenotype. We are currently addressing this possibility.

Cleavage of the M2 gene product precedes appearance of the ^{51}Cr release phenotype. To determine at what point during

FIG. 3. Reassortant GW524 M2 gene product μ 1/ μ 1C runs with an altered mobility relative to T1L and T3A μ 1/ μ 1C. Purified GW524 (lane 2), T1L (lanes 1 and 3), and T3A (lane 4) were boiled for 2 min in sample buffer, and approximately 4×10^{10} particles per lane were run on an SDS-10% polyacrylamide gel for 120 mA $\cdot$ h. The gel was then fixed, stained, destained, and dried as described in Materials and Methods. Viral proteins are labeled at the left, and the positions of marker proteins are designated by their M_r s (in thousands) at the right.

FIG. 4. Effect of the extent of virion digestion on ^{51}Cr release. [^{35}S]methionine-labeled T3A reovirus (at 10^{12} particles per ml) was digested with 200 μg of TLCK-treated α -chymotrypsin per ml at 37°C for the indicated amounts of time. Digestion reactions were stopped by adding phenylmethylsulfonyl fluoride to 2 mM, and the reaction mixtures were chilled to 4°C . (A) A 0.1-ml aliquot of each sample (5×10^5 particles per cell) was tested in the ^{51}Cr release assay (method A; see Materials and Methods). ^{51}Cr released into the medium as measured at 90 min postadsorption and the percent total release were calculated. Total release in this experiment was 11,101 cpm. (B) A 0.01-ml aliquot of each ISVP digest (10^{10} particles) was diluted in sample buffer, and the proteins were separated by SDS-PAGE and visualized by autoradiography as described in Materials and Methods. F, dye front.

the conversion of virion to ISVP the reovirus particle acquires the capacity to permeate cell membranes, we digested reovirus T3A particles at 37°C and used phenylmethylsulfonyl fluoride to interrupt the digestion process at various time points. An aliquot of the resulting particles then was either tested in the ^{51}Cr release assay or analyzed by SDS-PAGE. We found that by 2 min of digestion, particles had become capable of membrane interaction, as measured by rapid release of ^{51}Cr (Fig. 4A). At 2 min of digestion, most of the σ_3 protein had proteolytically degraded, and nearly half of the $\mu_1\text{C}$ had undergone cleavage to δ (Fig. 4B). The 16- and 24-kDa bands which are evident during the first minute of chymotrypsin digestion are probably σ_3 degradation products similar to V8 protease digestion products previously described (25). The spot in the second 2-min lane of Fig. 4B is an artifact.

In an attempt to determine the relative importance of σ_3 loss versus $\mu_1\text{C}$ -to- δ cleavage in appearance of the ^{51}Cr phenotype, we carried out the chymotrypsin digestion at 6°C . At this lower temperature, digestion was slowed down and particles acquired the ^{51}Cr release phenotype between

FIG. 5. Effect of the extent of virion digestion at 6°C on ^{51}Cr release. Experiments were performed as described in the legend to Fig. 4; however, the TLCK-treated α -chymotrypsin digestion was slowed by incubation at 6°C . (A) A 0.1-ml aliquot of each sample was tested in the ^{51}Cr release assay. ^{51}Cr released into the medium was measured at 60 min postadsorption, and the percent total ^{51}Cr release was calculated. (B) Samples were subjected to SDS-PAGE and visualized by autoradiography as described in the legend to Fig. 4. (C) The extent of protein digestion during conversion of virions to ISVPs was compared with the amount of ^{51}Cr released by the particles. The autoradiogram of the gel depicted in panel B was analyzed by densitometry, and the σ_3 , $\mu_1\text{C}$, and δ proteins were quantitated relative to the λ band, which remained constant during the course of digestion.

120 and 150 min of digestion (Fig. 5A). During this time, $\sigma 3$ was being degraded and $\mu 1C$ was undergoing cleavage to δ (the $\sigma 3$ degradation products in Fig. 4 were run off this gel) (Fig. 5B). Densitometry of the autoradiogram of the 6°C digestion was performed, and the amounts of $\sigma 3$, $\mu 1C$, and δ proteins relative to the λ proteins were determined. The ratios of $\sigma 3$, $\mu 1C$, and δ to λ were plotted in parallel with the ^{51}Cr release data (Fig. 5C). These data indicate that $\sigma 3$ itself is not directly involved in membrane interaction, since particles which have had greater than 99% of their $\sigma 3$ removed induce ^{51}Cr release. Greater than 50% of $\sigma 3$ was degraded prior to detection of ^{51}Cr release, suggesting that removal of $\sigma 3$ from a particle is insufficient to instill a ^{51}Cr release phenotype; however, this observation does not rule out the possibility that $\sigma 3$ removal is necessary to allow the ^{51}Cr release phenotype. For instance, removal of $\sigma 3$ may allow changes in the conformation of one or more outer capsid proteins or allow access to a previously masked proteolytic cleavage site. Cleavage of $\mu 1C$ to δ follows removal of $\sigma 3$ and precedes appearance of the ^{51}Cr release phenotype, indicating that this cleavage may be required for ISVP-induced ^{51}Cr release. However, the cleavage of $\mu 1C$ to δ and ϕ did not exactly correspond to the appearance of the ^{51}Cr release phenotype.

DISCUSSION

In this study, a ^{51}Cr release assay has been used to investigate the mechanism by which mammalian reovirus interacts with the cell membrane to gain access to the cytoplasm. We have determined that the reovirus M2 gene product, directly or indirectly, plays a role in the events which result in membrane permeabilization.

Unlike enveloped viruses, the nonenveloped animal viruses, by their nature, do not utilize membrane-to-membrane fusion as a means of gaining access to the cytoplasm of a host cell. These viruses might enter cells by insertion of a hydrophobic viral protein into the host cell membrane, thereby creating a channel through which substrate or nucleic acids could pass, or by disrupting the membrane sufficiently to allow the entire infectious particle to enter the cell. Membrane interaction could occur either at the cell surface, allowing direct penetration by the virus, or from within an endosome following receptor-mediated endocytosis.

Borsa et al. have previously demonstrated that T3A ISVPs generated *in vitro* possess the capacity to permeabilize cell membranes at the cell surface (2). It is possible that ISVPs generated in vesicles may permeabilize the endosome/lysosome membrane by an identical mechanism to gain access to the cytoplasm. The evidence that virion conversion to ISVP is a required step in the infectious process in that when virion-to-ISVP conversion within the endosome is prevented by treatment with ammonium chloride or protease inhibitors, infection is blocked (21, 28). ISVPs, on the other hand, are infectious in the presence of ammonium chloride and protease inhibitors (21, 28). Since permeabilization of L cells (at the cell surface) by *in vitro*-generated ISVPs may be representative of the events which occur within the endosome/lysosome during conventional reovirus entry, we have used the ^{51}Cr release assay to study early events in reovirus infection.

To determine which reovirus components are and are not involved in membrane interaction, different forms of reovirus were tested for the capacity to induce ^{51}Cr release. ISVPs generated from T3A virions and top component

(virions lacking their double-stranded RNA genome) induced ^{51}Cr release, demonstrating that neither $\sigma 3$ nor the RNA component of reovirus is required for membrane interaction (Fig. 1). Proteins which are on the ISVP surface (and therefore potentially interact with the cell membrane) include the $\lambda 2$ spike, the $\sigma 1$ attachment protein, and $\mu 1$ (the predominant fragments of $\mu 1$ found in ISVPs are δ and ϕ [20]). One or more of these viral proteins must directly and/or indirectly be involved in membrane interaction.

We undertook a genetic study in an attempt to identify a gene or genes involved in reovirus interaction with cell membranes. A reovirus strain difference in the ^{51}Cr release phenotype was identified. T1L ISVPs were found to be less efficient than T3A ISVPs at inducing release of ^{51}Cr from preloaded cells (Fig. 2). Reassortant analysis indicated that the M2 gene segment of T3A was associated with efficient membrane perturbation by reovirus (Table 3). The M2 gene segment encodes the $\mu 1$ protein, which undergoes cleavage to $\mu 1N$ and $\mu 1C$ during assembly; $\mu 1C$ undergoes cleavage to δ and ϕ during virion conversion to ISVP (20).

Since ISVPs possess the capacity to induce ^{51}Cr release, we favor the possibility that the predominant form of $\mu 1$ present in ISVPs, represented by fragments δ and ϕ , is the form of the protein involved in the membrane interaction role ascribed to the M2 gene product by the genetic studies described above. Although it is clear that the $\sigma 3$ protein is not required for ^{51}Cr release, we have not ruled out the possibility that the presence of this protein inhibits events, such as conformational changes in, or proteolytic cleavage of, one or more reovirus proteins required for ^{51}Cr release. Alternatively, $\sigma 3$ may mask viral components which directly interact with the membrane during permeabilization.

The study presented here indicates that the reovirus M2 gene product plays a role in reovirus interaction with the cell membrane. It is possible that the effect of the M2 gene on ^{51}Cr release is an indirect one and that other proteins on the surface of ISVPs ($\lambda 2$ or $\sigma 1$) are involved. In this case, the M2 gene product could alter conformation of the other capsid proteins. We favor the other possibility, that the M2 product plays a direct role. While the M2 gene product, $\mu 1$, does not contain any recognizable fusogenic sequences (13), it does contain a number of amphipathic α helices, two of which border the δ - ϕ cleavage site and have been proposed to play a role in membrane interaction (20). Amphipathic α helices have been suggested to play a role in membrane penetration for a number of enveloped and nonenveloped viruses (9, 29, 30). In addition, the ϕ fragment of $\mu 1$ is extremely basic (pH ≈ 11) and has been proposed to play a role in membrane penetration (20), as has the myristoyl group at the amino terminus of $\mu 1$ (22). Studies in other systems, such as poliovirus, have demonstrated other capsid proteins which are myristoylated (6, 23) and have suggested a possible role for these proteins in membrane interaction and virus entry. The fact that the ^{51}Cr release phenotype did not correspond precisely with the cleavage of $\mu 1C$ to δ and ϕ could indicate that further changes (i.e., conformational) might be occurring prior to interaction with cell membranes. Further studies will be needed to examine this point.

We are currently performing genetic and immunological studies which seek to define specific regions of the $\mu 1$ protein that may be involved in ISVP interaction with the cell membrane. Illumination of the $\mu 1$ protein's role in membrane interaction, whether direct or indirect, should allow us to provide a clearer model as to how reovirus delivers its transcriptional program to the cytoplasm.

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