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Mutations in Type 3 Reovirus That Determine Binding to Sialic Acid Are Contained in the Fibrous Tail Domain of Viral Attachment Protein σ1

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The reovirus attachment protein, σ1, determines numerous aspects of reovirus-induced disease, including viral virulence, pathways of spread, and tropism for certain types of cells in the central nervous system. The σ1 protein projects from the virion surface and consists of two distinct morphologic domains, a virion-distal globular domain known as the head and an elongated fibrous domain, termed the tail, which is anchored into the virion capsid. To better understand structure-function relationships of σ1 protein, we conducted experiments to identify sequences in σ1 important for viral binding to sialic acid, a component of the receptor for type 3 reovirus. Three serotype 3 reovirus strains incapable of binding sialylated receptors were adapted to growth in murine erythroleukemia (MEL) cells, in which sialic acid is essential for reovirus infectivity. MEL-adapted (MA) mutant viruses isolated by serial passage in MEL cells acquired the capacity to bind sialic acid-containing receptors and demonstrated a dependence on sialic acid for infection of MEL cells. Analysis of reassortant viruses isolated from crosses of an MA mutant virus and a reovirus strain that does not bind sialic acid indicated that the σ1 protein is solely responsible for efficient growth of MA mutant viruses in MEL cells. The deduced σ1 amino acid sequences of the MA mutant viruses revealed that each strain contains a substitution within a short region of sequence in the σ1 tail predicted to form β-sheet. These studies identify specific sequences that determine the capacity of reovirus to bind sialylated receptors and suggest a location for a sialic acid-binding domain. Furthermore, the results support a model in which type 3 σ1 protein contains discrete receptor binding domains, one in the head and another in the tail that binds sialic acid.

The initial step in productive viral infection is stable attachment of the virion to a cell surface receptor, typically protein or carbohydrate, capable of mediating entry of the intact virion or its genome into the intracellular environment (reviewed in reference 38). Reovirus attachment protein σ1 has emerged as an extremely useful and relevant model for the study of functional organization within a viral attachment protein. The σ1 protein is the determinant of the serotype-specific neutralizing antibody response (34) and is responsible for serotype-specific patterns of reovirus neuropathogenesis in newborn mice (31, 33, 35). Unique patterns of disease attributable to σ1 are determined by differences in the receptor specificity of σ1 proteins from different virus strains (8, 28). The σ1 protein projects from the 12 vertices of the virion capsid and assumes the morphology of an elongated fibrous domain, the tail, that terminates in a virion-distal globular domain, the head (13). Predictions of secondary structure indicate that repeat patterns of apolar amino acids in the tail contribute to formation of tandemly arranged regions of α-helical coiled-coil and cross β-sheet (12, 19). A considerably more complex mixture of secondary structures is proposed for the σ1 head (12, 19). Domains in σ1 demarcated by major transitions in proposed secondary structure correlate well with discrete morphologic domains identified in electron microscopic images of σ1 isolated from virions (12).

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Evidence exists for receptor-binding domains in both the head and tail of σ1. Genetic and biochemical studies indicate that the head of type 3 σ1 contains sequences important for binding receptors on diverse cell types, including murine L929 (L) cells (11, 18, 30, 37, 39), murine thymoma cells (6, 26, 37), rat neuroblastoma cells (6), explanted mouse and rat cortical neurons (9), and cells in certain regions of the murine central nervous system (27). The tail has been implicated in receptor binding on L cells (18), mammalian erythrocytes (7, 17), and murine erythroleukemia (MEL) cells (25), an erythroid precursor cell line permissive for infection by type 3 but not type 1 reovirus strains (25). Studies of hemagglutination (HA) activity and MEL cell infectivity among a panel of type 3 reovirus strains revealed a correlation between sequence polymorphism in the σ1 tail and the capacity to bind receptors on erythrocytes (7) and MEL cells (25). These changes clustered within a single predicted β-strand, suggesting that this region of sequence participates in the formation of a receptor-binding domain.

Terminally sialylated oligosaccharides serve as binding moieties on erythrocytes for type 3 reovirus (1, 21). Since HA-negative reovirus strains manifest a markedly reduced capacity to bind and infect MEL cells (25), it is likely that sialic acid is an essential receptor component on these cells. Thus, a model for receptor binding by type 3 σ1 holds that interaction of sequences in the tail with sialic acid is required for productive infection of some types of cells. This model is supported by studies of certain reovirus strains in which disassembly intermediates containing cleaved σ1 proteins were found to be capable of binding sialylated receptors on erythrocytes and infecting murine L cells by a sialic acid-dependent mechanism

(5, 18). Although these findings implicate the tail in binding of sialylated receptors, the precise location of the sialic acid-binding domain in σ1 remains undetermined. Furthermore, it is not known whether variable amino acid residues in the σ1 tail that correlate with binding of sialylated receptors are independent determinants of this property or exert their effects on receptor binding only in the context of additional sequence variability elsewhere in σ1.

To better define the mechanism of reovirus attachment to cells, three HA-negative strains of type 3 reovirus were adapted to growth in MEL cells to select mutations that promote viral binding to sialylated cellular receptors. Reassortant genetics was used to identify viral genes responsible for sialic acid-dependent adaptation of reovirus growth, and deduced amino acid sequences of mutant genes were determined to elucidate the molecular basis for sialic acid binding. Our results allow us to identify specific residues in σ1 that determine the capacity of reovirus to bind sialylated receptors. Furthermore, the findings support a model of topologically distinct receptor-binding domains in σ1 protein (18).

MATERIALS AND METHODS

Cells and viruses. Spinner-adapted L cells were grown in either suspension or monolayer cultures in Joklik's modified Eagle's minimal essential medium (Irvine Scientific, Santa Ana, Calif.) that was supplemented to contain 5% fetal bovine serum (Intergen, Purchase, N.Y.), 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin per ml (Irvine Scientific). Suspension cultures of MEL cells were grown in F-12 medium (Irvine Scientific) supplemented to contain 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, and 0.25 μg of amphotericin per ml. Reovirus strains type 1 Lang (T1L) and type 3 Dearing (T3D) are laboratory stocks. HA-negative strains type 3 clone 43 (T3C43), type 3 clone 44 (T3C44), and type 3 clone 84 (T3C84) were obtained from the collection of L. Rosen (7, 22–24). Purified virions were prepared by using second-passage L-cell lysate stocks of twice-plaque-purified reovirus as previously described (13).

Determination of viral titers after growth in L cells and MEL cells. Viral titer was determined by plaque assay (32). Following growth of virus for the desired interval, cells were frozen and thawed (−70 and 37°C) twice, and lysates were serially diluted 10-fold. Diluted virus was used to infect L-cell monolayers in duplicate in six-well plates (Costar, Cambridge, Mass.). L cells then were overlaid with medium 199 (Irvine Scientific) supplemented to contain 2.5% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 1% agar (Difco, Detroit, Mich.). Monolayers were stained with neutral red (Fisher Scientific, Pittsburgh, Pa.) and counted on day 7.

Adaptation of HA-negative type 3 reovirus strains to growth in MEL cells. Independent cultures of MEL cells (2.5×10^6 cells) were inoculated with a second-passage stock of reovirus strains T3C43, T3C44, and T3C84 at a multiplicity of infection (MOI) of 50 in a total volume of 500 μl, followed by virus adsorption at room temperature for 1 h. Cells then were diluted in growth medium to 5×10^5 cells per ml and incubated at 37°C for 7 days. Cultures were frozen and thawed twice, and 200 μl of culture lysate were used to infect a fresh aliquot of cells. This procedure was repeated for each HA-negative strain for a total of 10 passages.

Isolation of MA mutant viruses. MEL-adapted (MA) mutant viruses derived from serial passage of T3C43, T3C44, and T3C84 in MEL cells were isolated by plaque purification on MEL cells. Tenth-passage stocks were diluted 10-fold serially and used to infect monolayers of MEL cells in six-well plates. A previously described method to promote adherence of MEL cells was adapted to prepare confluent MEL cell monolayers (14). Plastic dishes were treated with a solution of 2.5% glutaraldehyde (Sigma Chemical Co., St. Louis, Mo.) at room temperature for 2 h, followed by several washes with water to remove excess glutaraldehyde. Plates then were coated with 200 μl of a 2.5-mg/ml solution of concanavalin A (Sigma) and allowed to dry at 37°C overnight. Plates were washed several times with phosphate-buffered saline (PBS; 140 mM NaCl, 2.7 mM KCl, 8.3 mM Na_2HPO_4, 1.5 mM KH_2PO_4), and 4×10^6 cells were deposited into each well. After 2 to 4 h of incubation at 37°C, cells were infected with virus and processed according to the plaque assay technique (32). The medium used to overlay MEL cells in monolayer consisted of an F-12 base supplemented to contain 10% fetal bovine serum, 2 mM L-glutamine, 100 U of penicillin per ml, 100 μg of streptomycin per ml, 0.25 μg of amphotericin per ml, and 1% agar. MA mutants were plaque purified twice and amplified in MEL cell cultures (6×10^6 cells) to generate second- and third-passage stocks of viral lysates.

Evaluation of viral growth in MEL cells and L cells. Yields of parental and MA mutant viruses in MEL cells and L cells were determined following viral growth at 37°C for 24 h. A total of 5×10^5 cells in a volume of 200 μl of gelatin saline (32) were adsorbed with a second-passage virus stock at an MOI of 1 PFU per cell at room temperature for 1 h. Excess virus was removed by washing cells with 1 ml of growth medium. Cells then were resuspended in 1.5 ml of medium and incubated.

Assessment of viral receptor binding by fluorescence-activated cytometric analysis. Binding of MA mutant and parental strains to MEL cells was analyzed as previously described (25). Briefly, 10^6 MEL cells were adsorbed with 5×10^{10} particles of purified virus, followed by the addition of a 1:20 dilution of rabbit polyclonal serum raised to strain T3D (Cocalico, Reamstown, Pa.). Cells then were incubated with fluorescein isothiocyanate-conjugated $F(ab')_2$ of goat anti-rabbit immunoglobulin G (Sigma) and analyzed with a FACS IV flow cytometer (Becton Dickinson Co., San Jose, Calif.). A positive signal for receptor binding was designated to be that in excess of fluorescence produced by primary and secondary antibodies in the absence of virus.

HA assay. Purified virions of MA mutant viruses or control strains were serially diluted twofold in 0.05 ml of PBS beginning with 10^{11} particles in 96-well round-bottom microtiter plates (Costar). Type O human erythrocytes or calf erythrocytes (Colorado Serum Co., Denver, Colo.) were washed twice in PBS and resuspended at a concentration of 1% (vol/vol). Erythrocytes (0.05 ml) were added to wells containing virus and incubated at 4°C for at least 2 h. The smallest number of viral particles sufficient to produce HA was designated 1 HA unit. The HA titer was defined as the number of HA units per 10^{11} viral particles.

Determination of the effect of neuraminidase treatment of MEL cells on viral growth. Terminal sialic acid residues were removed from cell surface carbohydrates by incubating 2×10^6 MEL cells at 37°C for 0.5 or 1.5 h in 1.0 ml of gelatin saline containing 2.5 to 100 mU of *Arthrobacter ureafaciens* neuraminidase (Sigma). Cells were washed in growth medium to remove neuraminidase and resuspended in aliquots of 5×10^5 cells in 200 μl of gelatin saline. Cells then were adsorbed with a second-passage stock of viral lysate at an MOI of 0.1 or 1.0 PFU per cell at room temperature for 1 h. Excess virus was removed by washing cells with 1.0 ml of growth medium, followed by resuspension of cells in 1.0 ml of medium. Cultures were incubated at 37°C for 18 h.

Isolation of reassortant viruses and identification of genes responsible for efficient infectivity of MA mutant viruses in MEL cells. Reassortant viruses were isolated by coinfecting monolayers of 3×10^6 L cells with either T1L and the MA mutant virus, T3C44-MA, or T1L and the parental strain, T3C44. Cells were inoculated at an MOI of 10 PFU per cell with either second- or third-passage stocks of parental strains in type 1/type 3 ratios ranging from 3:7 to 7:3. Cultures were incubated at 37°C for 3 days, followed by two cycles of freezing and thawing to release progeny virions. Reassortant viruses were twice plaque purified on L-cell monolayers and amplified in L cells.

Genotypes of viral reassortants were determined by analysis of viral genomic RNA extracted from second-passage stocks and resolved in sodium dodecyl sulfate–10% polyacrylamide gels. Viral RNA was purified by two to three phenol-chloroform (1:1) extractions of approximately 200 μl of second-passage virus stock, followed by ethanol precipitation. RNA-containing pellets then were reconstituted in sample buffer and electrophoresed at 25-mA constant current for 16 h. Gene segments were visualized by UV irradiation after staining with ethidium bromide.

Sequence analysis of MA mutant viral genes. The σ1-encoding S1 gene cDNAs of T3C43-MA, T3C44-MA, T3C84, and T3C84-MA were generated by using a previously described reverse transcription (RT)-PCR technique (15) and cloned into the pCR2.1 vector (Invitrogen, San Diego, Calif.). S1 genes were amplified with primers specific for the noncoding regions of the T3D S1 gene. Nucleotide sequences of cloned S1 gene cDNAs were determined by using phage T7 DNA polymerase (U.S. Biochemical, Cleveland, Ohio), S1-specific primers, and [^{35}S]ATP.

Nucleotide substitutions found in the cloned S1 gene cDNAs of MA mutant viruses were confirmed by sequence analysis of either S1 genomic double-stranded RNA (3) or RT-PCR products generated by using genomic viral RNA (15). RT-PCR products were resolved in 1% agarose–Tris-borate-EDTA gels stained with ethidium bromide. The S1 cDNA band was allowed to migrate onto DE-81 chromatography paper (Whatman, Maidstone, England) inserted into the gel, followed by elution in a solution of 20% ethanol, 1 M LiCl, 10 mM Tris (pH 7.6), and 1 mM EDTA. Eluted DNA was concentrated by ethanol precipitation and used as template in sequencing reactions with phage T7 DNA polymerase (U.S. Biochemical), S1-specific primers, and [^{35}S]ATP.

RESULTS

Adaptation of HA-negative reovirus strains to growth in MEL cells. Reovirus strains T3C43, T3C44, and T3C84 were serially passaged in MEL cells to select viral variants capable of efficient growth in these cells (Fig. 1). In a prior study, we found that yields of these strains in MEL cells are markedly lower than those of HA-positive type 3 strains (25). Upon serial passage, rapid selection occurred in which titers of all three HA-negative strains had increased by the first passage.

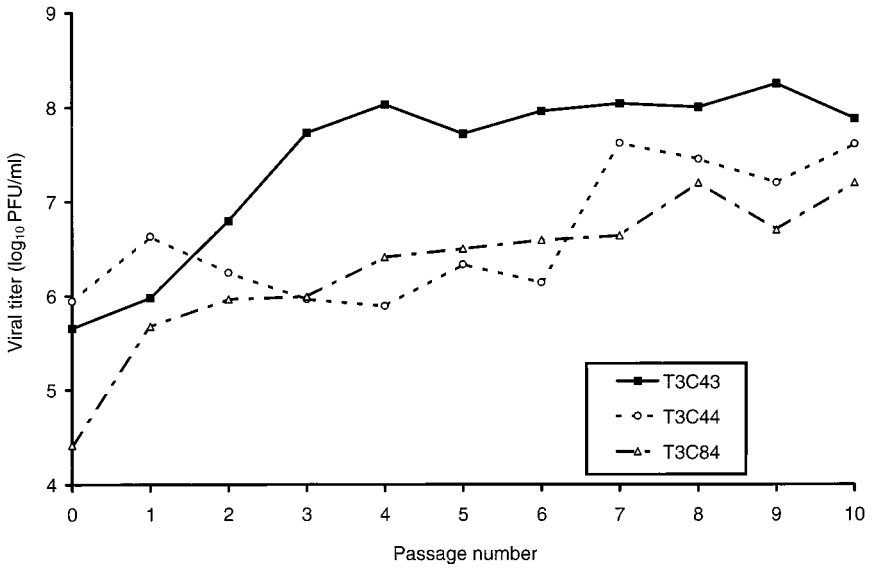


FIG. 1. Selection of reovirus mutants capable of efficient growth in MEL cells. Cultures of MEL cells (2.5×10^6) were infected with a second-passage lysate stock of type 3 reovirus strains T3C43, T3C44, and T3C84 at an MOI of 50 PFU per cell. Cultures were incubated at 37°C for 7 days and then lysed by twice freezing and thawing. A 200-μl aliquot of culture lysate was used to infect a fresh culture of MEL cells, and the process was repeated for a total of 10 passages. At each passage, virus contained in culture lysates was titrated by plaque assay using L-cell monolayers. MA mutant viruses were isolated from passage 10 stocks by being plaque purified twice on monolayers of MEL cells.

This trend continued until a general stabilization in titer occurred by passage 4 in the case of T3C43 and passage 7 in the cases of T3C44 and T3C84. MA mutant viruses were cloned from passage 10 stocks by plaque purification on MEL cells, and a single viral clone from MA stocks of T3C43, T3C44, and T3C84 was selected for characterization. Serial passage of the HA-positive prototype strain, T3D, in MEL cells produced viral titers of greater than $10^{6.5}$ PFU per ml through passage 8 followed by a precipitous decline to a titer of approximately 10^4 PFU ml (data not shown). This result is similar to a previous finding that serial passage of T3D in L cells resulted in a decline in viral titer, owing to the accumulation of defective interfering particles (4).

Growth of MA mutant viruses in MEL cells and L cells. To quantitatively characterize the growth phenotype of the MA mutants, yields of T3C43-MA, T3C44-MA, and T3C84-MA in MEL cells and L cells were compared with those of the parental strains after 24 h of viral growth (Fig. 2). MA mutant viruses grew to significantly higher titers in MEL cells, with yields ranging from 30- to 130-fold over those of the respective parental viruses. Titers well below the input inoculum (less than $10^{4.5}$ PFU per ml) were recorded for the parental strains following the same period of incubation. Yields of parental and MA mutant viruses in L cells were approximately the same, with differences of three- to fivefold, depending on the particular wild type-mutant pair. Thus, adaptation of HA-negative viruses to growth in MEL cells results in selection of mutants that efficiently infect these cells and does not appear to compromise the capacity of these viruses to grow in L cells. The latter property is consistent with a previous observation that

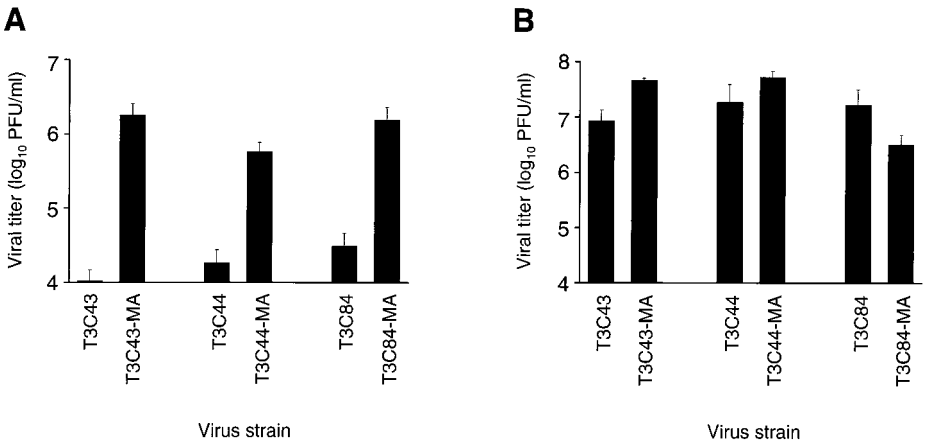


FIG. 2. Growth of parental and MA mutant viruses in MEL cells (A) and L cells (B). Suspension cultures of MEL cells (5×10^5) or L cells (5×10^5) were adsorbed with a second-passage lysate stock of HA-negative type 3 reovirus or MA mutant virus at an MOI of 1 PFU per cell. After 1 h of viral adsorption, cells were washed to remove unadsorbed virus and incubated at 37°C for 24 h. Cells were disrupted by two freeze-thaw cycles, and virus contained in lysates was titrated by plaque assay using L-cell monolayers. Viral yields are expressed as the means for four independent experiments. Error bars indicate standard deviations of the means.

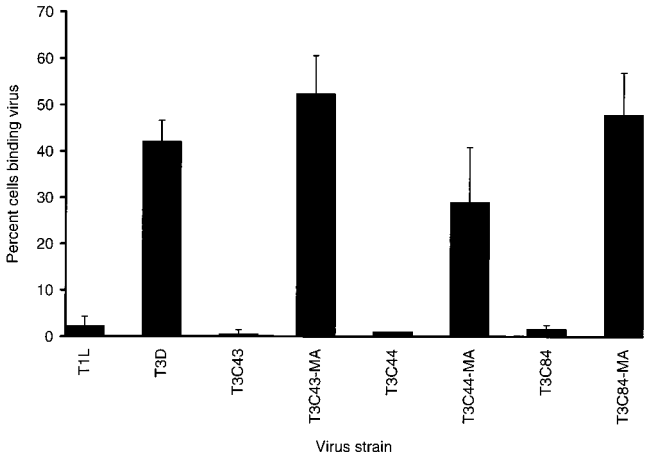


FIG. 3. Binding of parental and MA mutant viruses to MEL cells. MEL cells (10^6) were adsorbed with 5×10^{10} purified virions of HA-negative type 3 reovirus or MA mutant strains. Adsorbed virus was incubated with rabbit antireovirus antiserum to strain T3D, followed by FITC-conjugated goat anti-rabbit antibody. The amount of cell fluorescence was determined with a fluorescence-activated flow cytometer. Percent cells binding virus represents the fraction of cells demonstrating fluorescence intensity greater than the level of cells treated with primary and secondary antibodies alone. Each data point represents the mean fluorescence for four binding assays. Error bars indicate standard deviations of the means.

type 3 reovirus strains differing in the capacity to infect MEL cells produce equivalent yields in L cells (25). Additional clones of MA mutant viruses isolated from earlier times of adaptation (passages 4, 6, and 7) demonstrated growth characteristics in L cells and MEL cells very similar to those of the passage 10 isolates (data not shown); thus, growth phenotypes of the cloned passage 10 MA mutants appear to be representative of the MA mutant virus population.

Binding of MA mutant viruses to MEL cells. To determine whether adaptation of MA mutant viruses to growth in MEL cells had been achieved by an augmented viral binding capacity, we used fluorescence-activated cytometry to compare binding of parental and MA mutant viruses to MEL cells (Fig. 3). From 30 to 50% of cells incubated with MA mutant viruses were positive for viral binding, whereas 2% or less of cells adsorbed with the parental virus strains were positive for bound virus. The amount of viral binding exhibited by MA mutant strains paralleled that of T3D, which grows well in MEL cells (25). In contrast, binding by the HA-negative parental strains mirrored binding by T1L, which does not grow in MEL cells (25). Hence, binding of MA mutant viruses to MEL cells approximates that of an HA-positive type 3 strain, which indicates that growth of MA mutant viruses in MEL cells correlates with viral attachment.

HA activity of MA mutant viruses. The finding that HA-negative type 3 reovirus strains do not grow in MEL cells (25) suggested that variants selected for growth in these cells have acquired the capacity to bind sialic acid-containing virus receptors. Therefore, MA mutant strains were evaluated for the capacity to bind sialylated receptors in HA assays using mammalian erythrocytes (Fig. 4). In contrast to the parental strains, which did not agglutinate either human or bovine erythrocytes at the highest concentration of virus tested (10^{11} particles), MA mutant viruses efficiently agglutinated both types of erythrocytes. HA titers ranged from 32 to 2,048, which approximated or exceeded the HA titer of HA-positive control strain, T3D. Hence, HA-negative viruses adapted to growth in MEL cells acquired the capacity to efficiently produce HA, which suggests that MA mutant viruses have gained the capacity to bind sialic acid. Consistent with HA patterns described previously for type 3 reovirus strains (7), MA mutant viruses agglutinated human erythrocytes less efficiently than control strain T1L. T1L does not bind sialic acid (20) and likely agglutinates human erythrocytes by a mechanism different from that of type 3 reovirus strains. T1L was shown previously to lack HA activity with bovine erythrocytes (7).

Requirement of sialic acid for reovirus infectivity in MEL cells. Because the capacity of type 3 reovirus strains to infect MEL cells was found to vary with the capacity to mediate HA, we believed it probable that binding of sialic acid by MA mutant viruses would be required for their growth in MEL cells. However, we also thought that passage of virus in MEL cells could have selected additional mutations that facilitate infection by a mechanism independent of sialic acid binding. To determine the role of sialic acid in the infection of MEL cells by type 3 reovirus, we used *A. ureafaciens* neuraminidase to remove sialic acid from cell surface glycoconjugates prior to infection with prototype strain T3D (Fig. 5A). After 18 h of viral growth in neuraminidase-treated cells, yields of T3D were reduced approximately 10-fold at the lowest concentration of neuraminidase tested (2.5 mU per ml) and did not significantly decrease further at higher enzyme concentrations (up to 100 mU per ml). When the duration of neuraminidase treatment was extended from 0.5 to 1.5 h and viral MOI was reduced from 1 to 0.1 PFU per cell, virtually the same proportional decreases in viral yields were observed. The MA mutant viruses then were assessed for growth in MEL cells treated with neuraminidase (Fig. 5B). As with T3D, yields of MA mutant viruses in MEL cells after 18 h of growth were reduced by treatment of cells with neuraminidase. Approximately 10- to 75-fold reductions relative to untreated cells were observed at a concentration of 20 mU of neuraminidase per ml. These results demonstrate that sialic acid is a critical component of the MEL cell receptor for MA mutant viruses and that binding of sialylated receptors by the MA mutants is an essential property of their adaptation to growth in MEL cells.

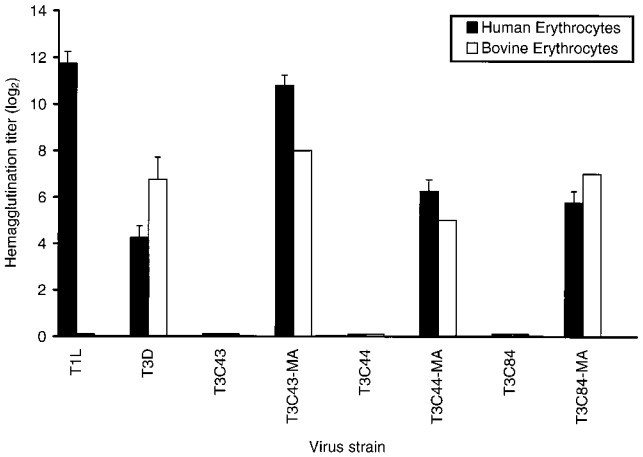


FIG. 4. HA activity of MA mutant viruses. Purified virions of parental and MA mutant viruses in PBS were serially diluted twofold from 1.0×10^{11} to 5×10^7 particles in 96-well microtiter plates. Either human type O or bovine erythrocytes were added to wells containing virus, followed by incubation at 4°C for at least 2 h. The smallest number of viral particles sufficient to produce HA was designated 1 HA unit. The HA titer is defined as the number of HA units per 10^{11} particles. HA activities of prototype strains T1L and T3D are shown as controls. Titers represent the means for four or five assays. Error bars indicate standard deviations of the means.

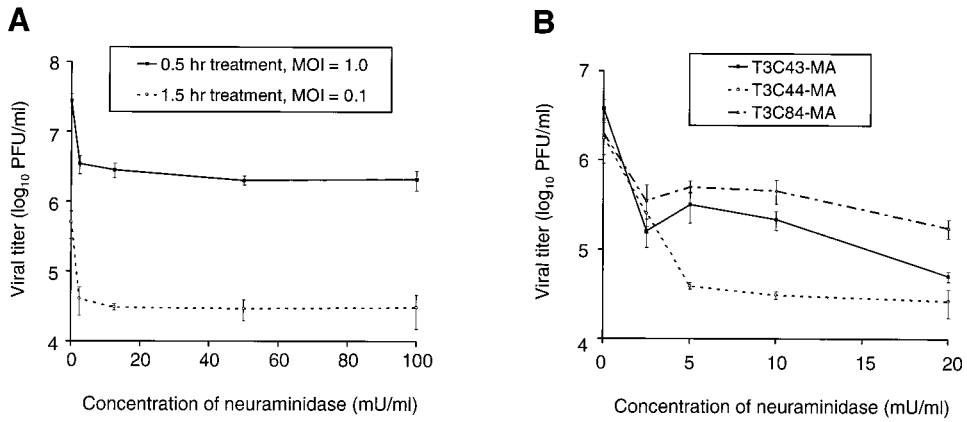


FIG. 5. Effect of neuraminidase on the infectivity of type 3 reovirus in MEL cells. Suspension cultures of MEL cells (5×10^5) were incubated with 2.5 to 100 mU of *A. ureafaciens* neuraminidase per ml at 37°C for 0.5 or 1.5 h prior to adsorption with a second-passage lysate stock of virus at an MOI of 0.1 or 1 PFU per cell. Cells were incubated at 37°C for 18 h and then processed for plaque assay using L-cell monolayers. Viral yields are expressed as the means for four independent experiments. Error bars indicate standard deviations of the means. (A) Infectivity of HA-positive prototype strain T3D. (B) Infectivity of mutant strains T3C43-MA, T3C44-MA, and T3C84-MA (1.5 h of treatment with neuraminidase, MOI of 0.1 PFU per cell).

Identification of viral genes associated with growth of MA mutant viruses in MEL cells. Sialic acid-dependent infection of MEL cells by MA mutant viruses strongly suggested that mutations in the σ1 proteins of these strains were responsible for efficient viral growth in MEL cells. Genetic (20, 25) and biochemical (21) studies of reovirus receptor binding indicate that σ1 is the principal, if not sole, viral protein that binds sialylated cellular receptors. However, we thought it conceivable that mutations selected in outer-capsid proteins adjacent to σ1 (e.g., λ2 or σ3 [10]) could constrain σ1 to a different conformation having increased affinity for sialic acid. Thus, to identify viral genes responsible for efficient growth of MA mutant strains in MEL cells, we isolated reassortant viruses by plaque purification from mixed infections of T3C44-MA and T1L, which does not grow in MEL cells (25). MEL cells were infected with 10 independent T3C44-MA × T1L reassortant viruses, and yields were compared to those of the parental strains (Table 1). Following 24 h of viral growth, T3C44-MA × T1L reassortant viruses separated into two distinct groups based on yields: those that achieved titers of $10^{4.6}$ to $10^{5.5}$ PFU per ml and those that grew to titers of $10^{7.5}$ to 10^8 PFU per ml. Viral titers among the former group of reassortants were similar to that of T1L ($10^{4.6}$ PFU per ml), whereas titers among the latter group approximated that of T3C44-MA ($10^{7.4}$ PFU per ml). When yields were correlated with the parental origins of viral genes, a clear association was observed between viral growth and derivation of the σ1-encoding S1 gene segment; all strains containing the T1L S1 gene produced titers of less than $10^{5.5}$ PFU per ml, whereas strains containing the T3C44-MA S1 gene grew to titers of greater than $10^{7.5}$ PFU per ml. No other viral gene segregated with this property. Therefore, these results provide genetic evidence that changes in the σ1 protein are responsible for adaptation of T3C44-MA to growth in MEL cells.

To exclude the possibility that growth of reassortant viruses containing the T3C44-MA S1 gene is due to novel combinations of T1L-derived and T3C44-derived genes, reassortant viruses were isolated from crosses of the HA-negative parental strain, T3C44, and T1L. MEL cells were infected with 10 independent T3C44 × T1L reassortant viruses, and yields were compared to those of parental strains (Table 2). None of the T3C44 × T1L reassortants grew significantly better than the parental strains, regardless of S1 gene derivation, and yields approximated those of T3C44-MA × T1L reassortants containing an S1 gene from T1L (Table 1). These results indicate that heterologous combinations of T3C44 and T1L genes do not confer the capacity for growth in MEL cells.

Changes in the σ1 amino acid sequences of MA mutant viruses. The molecular basis for growth of MA mutant viruses in MEL cells was explored by determining the deduced σ1 amino acid sequences of T3C43-MA, T3C44-MA, and T3C84-MA (Table 3). Sequence analysis of cloned S1 gene cDNAs from these strains revealed single amino acid substitutions in T3C43-MA σ1, $Asp^{198} \rightarrow Asn$, and T3C44-MA σ1, $Leu^{204} \rightarrow Pro$, in comparison to the parental strains. These substitutions occurred at positions that uniquely differ between the σ1 proteins of the HA-negative parental strains and a panel of type 3 HA-positive strains (7) (Fig. 6). Furthermore, the amino acid replacements resulted in the specific residues found at those

TABLE 1. Growth of T1L × T3C44-MA reassortant viruses in MEL cells

Virus strain	Gene segment[a]										Yield in MEL cells[b] (log_{10} PFU/ml)
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
Parental											
T1L	L	L	L	L	L	L	L	L	L	L	4.6
T3C44-MA	M	M	M	M	M	M	M	M	M	M	7.4
Reassortant											
L44MA-23	L	M	M	M	L	M	M	M	L	L	8.1
L44MA-18	M	M	M	L	L	L	M	L	M	M	7.9
L44MA-17	L	M	M	M	L	M	M	M	M	M	7.9
L44MA-5	L	M	L	L	L	L	M	L	L	L	7.6
L44MA-10	L	L	L	L	L	L	L	L	L	M	5.5
L44MA-4	L	L	L	M	L	L	L	M	M	M	5.0
L44MA-25	M	M	M	M	M	M	L	M	M	L	4.9
L44MA-11	L	L	L	L	L	L	L	M	M	M	4.9
L44MA-20	L	M	L	M	M	M	L	L	L	M	4.7
L44MA-14	M	M	M	M	M	M	L	M	M	M	4.6

[a] Parental origin of each gene segment: L, gene segment derived from T1L; M, gene segment derived from T3C44-MA.

[b] MEL cells (5×10^5) were infected with virus strains at an MOI of 1 PFU per cell and incubated at 37°C for 24 h. Cells were frozen and thawed twice prior to being titrated on L-cell monolayers by plaque assay. Shown are the means for two independent experiments.

TABLE 2. Growth of T1L × T3C44 reassortant viruses in MEL cells

Virus strain	Gene segment[a]										Yield in MEL cells[b] (log_{10} PFU/ml)
	L1	L2	L3	M1	M2	M3	S1	S2	S3	S4	
Parental											
T1L	L	L	L	L	L	L	L	L	L	L	4.6
T3C44	44	44	44	44	44	44	44	44	44	44	4.7
Reassortant											
L44-9	L	44	L	L	L	L	44	L	44	L	5.5
L44-37	L	44	L	L	L	L	44	44	L	L	5.5
L44-6	44	44	44	44	44	44	44	44	L	L	5.4
L44-40	L	L	L	44	L	L	L	44	L	44	5.4
L44-33	44	44	44	44	L	44	44	44	44	L	5.3
L44-8	L	L	L	44	L	L	L	L	L	L	5.3
L44-38	L	L	L	44	L	L	44	L	L	L	5.2
L44-41	L	L	L	L	L	L	44	44	44	L	5.2
L44-34	L	44	44	L	L	L	44	44	44	L	5.1
L44-7	44	44	L	44	44	L	L	44	44	L	5.0

[a] Parental origin of each gene segment: L, gene segment derived from T1L; 44, gene segment derived from T3C44.

[b] MEL cells (5×10^5) were infected with virus strains at an MOI of 1 PFU per cell and incubated at 37°C for 24 h. Cells were frozen and thawed twice prior to being titrated on L-cell monolayers by plaque assay. Shown are the means for two independent experiments.

positions in all type 3 HA-positive strains studied previously (7). The σ1 protein of T3C84-MA contained two substitutions in comparison to T3C84 σ1, Lys^{26}→Ile and Trp^{202}→Arg. As with the other MA mutant σ1 proteins, T3C84-MA σ1 was mutated at the position distinguishing it from σ1 proteins of HA-positive strains, amino acid 202, and an arginine is found at position 202 in HA-positive viruses (7). Each mutation in σ1 resulted from a single nucleotide change, and no other nucleotide substitutions were found in the T3C44-MA or T3C84-MA σ1-encoding S1 gene cDNAs; a single noncoding change was found in the T3C43-MA S1 gene cDNA (Table 3). Authenticity of σ1 mutations was confirmed by direct sequence analysis of either S1 gene double-stranded RNA or S1 gene RT-PCR products. In addition, analysis of the S1 gene cDNA of T3C84 confirmed that the Lys^{26}→Ile substitution in T3C84-MA σ1 was not contained in the parental virus stock (data not shown). Thus, sequence analysis indicates that mutations are contained in the σ1 proteins of all three MA mutant viruses. Furthermore, there is a clustering of changes in a short region of sequence predicted to form a single β-strand in the σ1 tail, amino acids 198 through 204 (19).

TABLE 3. Mutations in the S1 genes and σ1 proteins of MA mutant viruses

Virus strain	Location of mutation[a]		GenBank accession no.
	S1 gene	σ1 protein	
T3C43-MA	585, G→A	—	U74293
	604, G→A	198, Asp→Asn	
T3C44-MA	623, T→C	204, Leu→Pro	U74292
T3C84-MA	89, A→T	26, Lys→Ile	U74291
	616, T→C	202, Trp→Arg	

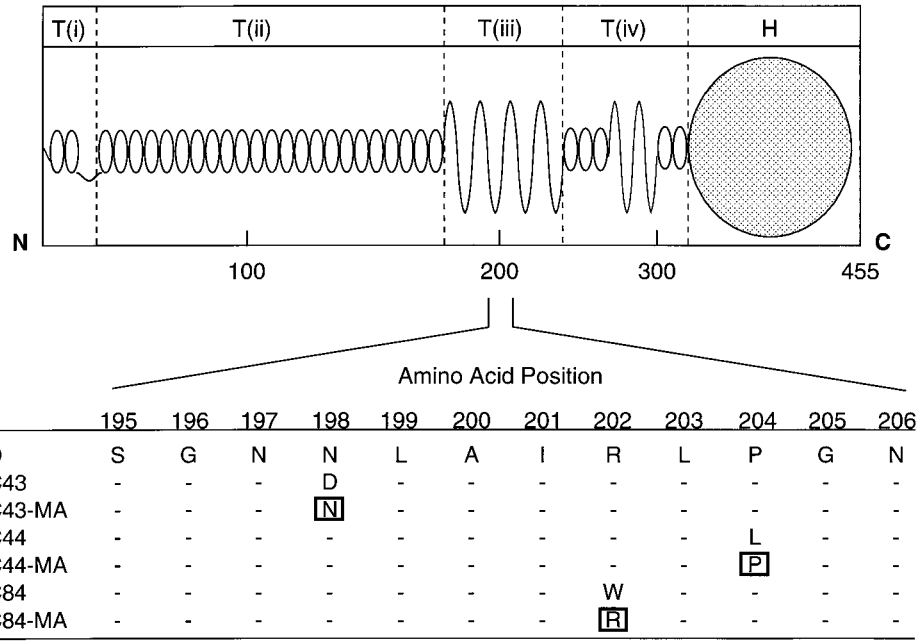
[a] Substitutions in the S1 genes and σ1 proteins are indicated by the corresponding nucleotide and amino acid positions, respectively. —, nucleotide change did not result in an amino acid substitution.

DISCUSSION

In this study, we selected reovirus variants containing point mutations in the attachment protein, σ1, that confer the capacity to bind sialylated cellular receptors. The alterations were selected by passaging viruses incapable of binding sialic acid in MEL cells, in which sialic acid is an essential component of the reovirus receptor. Mutations in the σ1 proteins of MA mutant viruses clustered within a seven-residue segment of predicted β-sheet in the σ1 tail, and no mutations were found in the head, which has been shown to contain a receptor-binding domain for L cells (11, 18, 30, 37, 39). These results provide evidence that a sialic acid-binding domain is contained in the tail of type 3 σ1. Furthermore, the approach of adapting reoviruses to utilize a new receptor represents a novel method for selecting mutant viruses for studies of viral attachment and structure-function relationships in σ1.

Previous studies suggested that sequences in type 3 σ1 important for binding sialic acid are contained within the tail (7, 17, 18, 25); however, specific residues in type 3 σ1 protein that determine the capacity of reovirus to recognize sialylated receptors have not been identified. Sequence variability at amino acid positions 198, 202, and 204 in the σ1 tail correlates with reovirus HA activity (7) and infectivity in MEL cells (25). One interpretation of sequence variability in the σ1 tail of HA-negative viruses is that these residues are important for the capacity of σ1 to bind sialic acid but manifested only in the context of additional differences between the σ1 proteins (or other viral proteins) of HA-negative and HA-positive strains. Results from the present study show that a single residue of T3C44 σ1, at position 204, determines the capacity of this virus to recognize sialylated receptors. The Leu^{204}→Pro substitution in T3C44-MA σ1 is the only difference between the T3C44 and T3C44-MA σ1 proteins, and genetic analysis of T3C44-MA × T1L reassortant viruses indicates that the T3C44-MA S1 gene, alone, is responsible for sialic acid-dependent growth of this strain in MEL cells (Table 1). Hence, other than position 204, variability among the σ1 proteins of T3C44 and HA-positive strains appears to be unrelated to viral binding of sialylated receptors. Since T3C43-MA also contains a single substitution in its σ1 protein compared to T3C43 σ1, Asp^{198}→Asn, it seems probable that the sole determinant of sialic acid binding by T3C43-MA is the specific amino acid located at position 198. However, confirmatory evidence will require genetic analysis of this mutant. Likewise, the Trp^{202}→Arg substitution in T3C84-MA σ1 occurs at the position in which the parental HA-negative strain uniquely differs from other HA-positive type 3 strains. Yet, the additional Lys^{26}→Ile change in T3C84-MA σ1 leaves open the possibility that the mutation at position 202 must be accompanied by one near the amino terminus to facilitate sialic acid binding. Nevertheless, these findings indicate that reovirus adaptation to binding sialic acid-containing receptors is tightly linked to a cluster of mutations within a short region of sequence corresponding to a single predicted β-strand in the σ1 tail (19). This β-strand represents the fourth in a series of eight consecutive β-strands proposed to form an amphipathic β-sheet structure (19). A model of σ1 structure suggests that the predicted β-sheet corresponds to morphologic domain T(iii) identified in negatively stained preparations of σ1 isolated from virions and visualized by electron microscopy (12). Morphologic domain T(iii) is contained entirely within the fibrous σ1 tail and is clearly distinguished from the carboxy-terminal globular domain representing the σ1 head (12).

The proximity and type of mutations selected in the variant σ1 proteins suggest that the short predicted β-strand contain-



	195	196	197	198	199	200	201	202	203	204	205	206
T3D	S	G	N	N	L	A	I	R	L	P	G	N
T3C43	-	-	-	D	-	-	-	-	-	-	-	-
T3C43-MA	-	-	-	[N]	-	-	-	-	-	-	-	-
T3C44	-	-	-	-	-	-	-	-	-	L	-	-
T3C44-MA	-	-	-	-	-	-	-	-	-	[P]	-	-
T3C84	-	-	-	-	-	-	-	W	-	-	-	-
T3C84-MA	-	-	-	-	-	-	-	[R]	-	-	-	-

FIG. 6. Sequence changes in the σ1 proteins of reovirus MA mutants. Mutations in the deduced σ1 amino acid sequences of MA mutant viruses are shown at their predicted locations based on a model of σ1 structure (19). Morphologic domains of σ1 [T(i), T(ii), T(iii), T(iv), and H] described previously are shown and scaled proportionally to the domains identified in electron microscopic images of σ1 isolated from virions (12). Amino acid positions are scaled according to their predicted relationship to individual σ1 morphologic domains (19). Amino acid sequences of the HA-negative parental strains and the HA-positive strain, T3D, are shown for comparison. Changes were not found at other positions in σ1 proteins of T3C43-MA and T3C44-MA. One additional change, $Lys^{26} \rightarrow Ile$, was observed in the σ1 protein of T3C84-MA.

ing those changes is very important for sialic acid binding. Because substitutions in this region resulted in the identical amino acids found at the homologous positions in HA-positive type 3 strains, residues in β-strand 4 of the proposed β-sheet may constitute an integral part of the ligand-binding site, possibly amino acids that coordinate with sialic acid itself. Hence, the simplest explanation for the attachment and growth properties of MA mutant viruses is that the observed changes in σ1 affect a critical interaction between these residues and sialic acid, perhaps stereochemical or charge related, leading to higher affinity between virus and cell. In this regard, it may be significant that residues 198, 202, and 204 of type 3 σ1 are predicted to reside on the exterior face of the amphipathic β-sheet structure suggested for morphologic domain T(iii) (19). Presentation of these residues in such a fashion might make them accessible to constituents of sialic acid. However, it is also possible that these amino acids interact with other surface-exposed residues in σ1, such as those contained in predicted β-loops, to stabilize the σ1 oligomer or maintain the framework of a sialic acid-binding cleft located elsewhere in the protein. Determination of the precise role of sequences in morphologic domain T(iii) will require higher-resolution models of σ1 structure.

Binding of sialylated receptors appears to be a strict requirement for reovirus infection of MEL cells (Fig. 5), which raises questions about the importance of sialic acid for growth of reoviruses in other cell types. Growth in L cells of the three HA-negative strains studied here is equivalent to that of the MA mutant viruses (Fig. 2). This result is in agreement with previous findings that HA-positive and HA-negative type 3 reovirus strains produce equivalent yields in L cells (25) and that the yield of T3D is not significantly diminished by pretreatment of L cells with neuraminidase (18). Thus, sialic acid is not a universal requirement for reovirus infection of cultured cells. Similarly, alterations that abrogate binding of sialylated receptors and apparently promote attachment and infection by sialic acid-independent mechanisms have been selected in other viruses, such as animal rotavirus (16, 40) and canine parvovirus (2, 29).

In a model of receptor binding by reovirus, the dispensability of sialic acid for reovirus infection of L cells is rationalized by evidence that σ1 protein contains a receptor-binding domain in the head that is specific for L-cell receptors and discrete from another domain that binds sialic acid (18). This model is supported by results from the present study showing that sequences in the tail determine the capacity of reovirus to bind sialylated receptors on MEL cells. Thus, the relative importance of receptor-binding domains in σ1 depends on the host cell. This pattern could be explained if L cells or MEL cells lack one type of receptor or another for independently functioning receptor-binding domains in the head and tail of σ1. However, current data do not exclude a model in which the σ1 head is not critical for viral attachment to MEL cells but is required for viral entry. This would be similar to a mechanism described for adenovirus entry in which the fiber and penton-base proteins mediate viral attachment and entry, respectively (36). The requirement for sialic acid in reovirus infection of cells other than L cells and MEL cells is unknown; however, utilization of this ubiquitous receptor in at least some cell types would be consistent with the diversity of cultured cells infected by reoviruses in vitro and the broad cell and tissue tropism of reoviruses in vivo. MA mutant viruses isolated in this study now make it possible to use isogenic reovirus strains that vary in sialic-acid binding to precisely define the role of this receptor in reovirus pathogenesis.

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