JOURNAL OF VIROLOGY, Oct. 1990, p. 5173–5176
0022-538X/90/105173-04$02.00/0

Vol. 64, No. 10

A σ1 Region Important for Hemagglutination by Serotype 3 Reovirus Strains

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Received 3 April 1990/Accepted 15 June 1990

Hemagglutination (HA) by the mammalian reoviruses is mediated by interactions between the viral σ1 protein and sialoglycoproteins on the erythrocyte surface. Three serotype 3 (T3) reovirus strains were identified that do not agglutinate either bovine or type O human erythrocytes (HA negative): T3 clone 43 (T3C43), T3 clone 44 (T3C44), and T3 clone 84 (T3C84). These three strains also showed a diminished capacity to bind the major erythrocyte sialoglycoprotein, glycophorin, in an enzyme-linked immunosorbent assay. To determine the molecular basis for these findings, we examined the deduced σ1 amino acid sequences of the three HA-negative T3 strains and four HA-positive T3 strains. The limited number of sequence differences in the σ1 proteins of these seven strains allowed us to identify single unique amino acid residues in each of the HA-negative strains (aspartate 198 in T3C43, leucine 204 in T3C44, and tryptophan 202 in T3C84) that cluster within a discrete region of the σ1 tail. The identification of σ1 residues important for HA and glycophorin binding suggests that tail-forming sequences are exposed on the virion surface, where they interact with carbohydrate residues on the surface of cells.

Many animal viruses, including the mammalian reoviruses, agglutinate erythrocytes in vitro (5, 21). In most cases, hemagglutination (HA) occurs through the interaction of virion surface proteins with carbohydrate groups attached to proteins on the erythrocyte surface (5). Reovirus strain type 3 Dearing (T3D) binds glycophorin, the major sialoglycoprotein on the surface of human erythrocytes (20). Although the biologic importance of HA is not known for most viruses, protein-carbohydrate interactions similar to those required for erythrocyte agglutination may play a role in viral receptor recognition during productive infection of nucleated cells. Binding to carbohydrate ligands on the cell surface is required for receptor recognition by influenza viruses (31) and may also play a role in recognition of L-cell receptors by reoviruses (1, 11, 12, 19, 20).

The reovirus hemagglutinin is the S1 translation product σ1 (6, 15, 20, 30). The σ1 protein is also the cell attachment protein (14, 28, 32) and is responsible for cell and tissue tropism (18, 25, 28, 29). Electron micrographs of σ1 suggest that the protein has the morphology of a fibrous tail (T) and a globular head (H) (2, 10). Recent studies of σ1 structure suggest that the protein is further subdivided into discrete morphologic domains [T(i), T(ii), T(iii), T(iv), and H] (9) that have distinct sequence characteristics (17). In this study, we examined the deduced σ1 amino acid sequences of a group of T3 reovirus strains that vary in their capacities to agglutinate erythrocytes and to bind glycophorin in order to investigate the structural basis for these σ1-associated functions.

We first investigated the capacity of 10 T3 field isolate strains (7, 13, 22–24) and the prototype strains T1 Lang (T1L) and T3D to agglutinate erythrocytes. Purified virions (10) were serially diluted twofold from 2.0×10^{11} to 1.0×10^{8} particles in 0.05 ml of cold phosphate-buffered saline and placed into 96-well round-bottom microtiter plates (Costar, Cambridge, Mass.). Either citrate-preserved bovine (Colorado Serum, Denver, Colo.) or type O human erythrocytes were washed twice in cold phosphate-buffered saline and suspended at a concentration of 0.8% (vol/vol). Erythrocytes (0.05 ml) were added to wells containing virus and incubated at 4°C for a minimum of 2 h. The smallest number of virion particles sufficient to produce HA was designated to equal 1 HA unit. The HA titer was defined as the number of HA units per 2×10^{11} particles, the largest number of virion particles used in these assays.

The 11 T3 strains examined in this study exhibited striking differences in their capacities to agglutinate bovine and human erythrocytes (Table 1). The HA titers of strains T3C43, T3C44, and T3C84 were <1 in assays using either bovine or human erythrocytes (HA negative). The HA titers of the other T3 strains were ≥128 in assays using bovine erythrocytes and ≥16 (with a single exception) in assays using human erythrocytes (HA positive). Agglutination of bovine erythrocytes has been suggested to be a property common to all serotype 3 reovirus isolates (8); however, Burstin et al. (6) observed that some T3 strains did not agglutinate bovine erythrocytes. Our findings concur that there are T3 strains that do not agglutinate bovine erythrocytes and indicate that these same strains do not agglutinate human erythrocytes either. Therefore, agglutination of bovine and human erythrocytes is not a property common to all T3 reovirus strains.

To determine whether the T3 strains also vary in their capacity to bind glycophorin, the major sialoglycoprotein on the surface of human erythrocytes, we used the method of K. L. Tyler and H. W. Virgin IV (unpublished data) to assess binding of virions to glycophorin in an enzyme-linked immunosorbent assay. Briefly, 96-well microtiter plates were coated overnight with purified virions and incubated in bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.)

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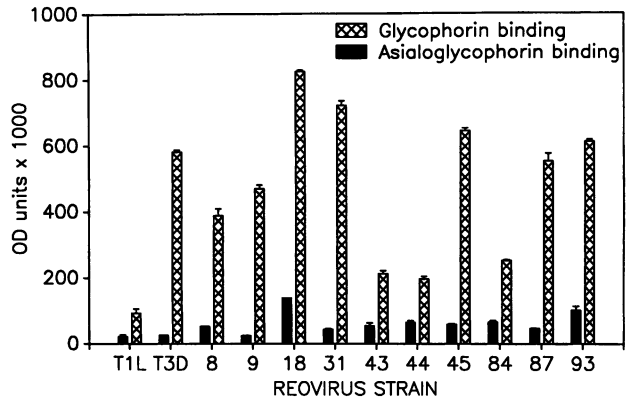

FIG. 1. Binding of reovirus strains to glycophorin. Binding of strains T1L, T3D, T3C8, T3C9, T3C18, T3C31, T3C43, T3C44, T3C45, T3C84, T3C87, and T3C93 (abbreviated by clone number) to biotinylated glycophorin or biotinylated asialoglycophorin was determined in an enzyme-linked immunosorbent assay. The mean ± standard deviation of triplicate determinations is shown for each strain. OD, Optical density.

according to the method of Virgin et al. (27). Plates were then incubated with either biotinylated glycophorin or biotinylated asialoglycophorin (human glycophorin; Sigma). Virus binding to biotinylated glycophorin or biotinylated asialoglycophorin was detected with peroxidase-conjugated streptavidin by using 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Sigma) as substrate. Optical density at 570 nm was determined by using an enzyme-linked immunosorbent assay reader (Dynatech Laboratories, Inc., Alexandria, Va.), and virus binding was expressed as optical density units × 1,000. The HA-negative T3 strains bound to glycophorin with less avidity than any of the HA-positive strains (Fig. 1). The relative binding of reovirus strains to human glycophorin correlated well with their HA titers in assays using bovine erythrocytes. Glycophorin binding by T1L (HA negative in assays using bovine, but not human, erythrocytes) was previously shown to be less than glycophorin binding by T3D (Tyler and Virgin, unpublished data). We now show that glycophorin binding by T1L is similar to glycophorin binding by the HA-negative T3 strains.

TABLE 1. HA titers of reovirus strains

Strain[a]	HA titer[b]	
	Bovine	Human
T1L	<1	512
T3D	512	16
T3C8	128	16
T3C9	256	32
T3C18	1,024	32
T3C31	1,024	32
T3C43	<1	<1
T3C44	<1	<1
T3C45	512	2
T3C84	<1	<1
T3C87	1,024	16
T3C93	1,024	16

[a] Reovirus strains included prototype strains T1 Lang (T1L) and T3 Dearing (T3D) and type 3 field isolate strains T3 clone 8 (T3C8), T3 clone 9 (T3C9), T3 clone 18 (T3C18), T3 clone 31 (T3C31), T3 clone 43 (T3C43), T3 clone 44 (T3C44), T3 clone 45 (T3C45), T3 clone 84 (T3C84), T3 clone 87 (T3C87), and T3 clone 93 (T3C93) (7, 13, 22, 23, 24). T3C87 is the T3 Abney strain (24).

[b] HA titer is expressed as 2×10^{11} particles divided by the number of particles per HA unit. One HA unit equals the number of particles sufficient to produce HA.

To identify amino acids in σ1 that might be associated with the σ1 properties of HA and glycophorin binding, we examined the σ1 amino acid sequences of seven closely related T3 strains (7) for residues unique to HA-negative strains (Fig. 2). The σ1 sequences of these seven T3 strains have differences at only 19 amino acid positions in the 455-amino-acid sequence. We found that the σ1 sequence of each HA-negative strain contains a single unique residue (aspartate 198 in T3C43, leucine 204 in T3C44, and tryptophan 202 in T3C84) and that these residues cluster within the σ1 tail. This analysis suggests that a discrete region of sequence within the σ1 tail is important for the capacity of T3 reovirus strains to agglutinate erythrocytes and to bind glycophorin. The single unique residues in HA-negative strains are located between amino acids 198 and 204 and are included in the region of sequence proposed to form morphologic region

Strain	AA: 22	53	88	99	167	195	198	202	204	236	244	245	246	249	255	264	329	356	408	HA
T3D	V	L	I	D	R	S	N	R	P	T	S	R	I	T	A	N	S	D	T	+
T3C43	I	L	T	D	R	S	(D)	R	P	T	S	R	I	I	A	N	S	D	A	-
T3C44	I	L	T	D	R	S	N	R	(L)	T	S	R	T	I	A	N	S	D	A	-
T3C45	I	L	T	D	R	L	N	R	P	A	S	R	I	I	T	D	S	D	A	+
T3C84	I	F	T	N	R	S	N	(W)	P	T	S	R	I	I	A	N	S	D	A	-
T3C87	I	L	T	D	R	S	N	R	P	T	S	G	I	I	A	N	S	N	A	+
T3C93	I	F	T	N	A	S	N	R	P	T	P	R	I	I	A	N	G	D	A	+

FIG. 2. Identification of residues important for HA of bovine and human erythrocytes. Amino acid differences between T3D, T3C43, T3C44, T3C45, T3C84, T3C87, and T3C93 are limited to the 19 nonconsecutive amino acid positions shown. Residues that are circled are unique to the σ1 amino acid sequences of HA-negative strains. Threonine 246 in T3C44 is not a unique residue among all of the T3 strains included in this study; amino acid differences are observed at position 246 in the HA-positive strains T3C9, T3C18, and T3C31 (7).

T(iii) in the σ1 tail (17). A long conserved region (amino acids 185 to 230) was identified within this morphologic region of σ1 in a comparison of σ1 protein from 11 T3 strains (7). Four amino acids were changed in this long conserved region: the single unique residues in the three HA-negative strains and leucine 195 in T3C45. While the T3C45 HA titer in assays using bovine erythrocytes was not appreciably different from other HA-positive strains, its HA titer in assays using type O human erythrocytes was eightfold lower than that of any other HA-positive strain (Table 1). Leucine 195 in T3C45 is adjacent to the region of sequence identified as important for HA and could effect a change in σ1 conformation in this region. Such a change could conceivably affect lattice formation required for the agglutination of human erythrocytes by T3C45 without affecting its binding to glycophorin (Fig. 1). Alternatively, the difference in HA by T3C45 in assays using bovine and human erythrocytes (Table 1) may reflect differences in the accessibility and/or chemical nature of the carbohydrate residues on these two cell types that are bound by σ1.

The region of sequence in T3 σ1 identified as important for HA and glycophorin binding corresponds to a single β-strand within a proposed large amphipathic β-sheet region in the σ1 tail (17). In the model for σ1 oligomerization proposed by Nibert et al. (17), apolar residues that form one face of the β-sheet provide intersheet hydrophobic interactions to create a dimeric cross-β sandwich. As shown in Fig. 3, unique amino acids in HA-negative strains would be part of the hydrophilic face of the β-sheet and might be exposed at the protein surface where they could function in HA and glycophorin binding. Alternatively, these residues could affect HA and glycophorin binding through a more indirect mechanism (e.g., by altering the conformation of a σ1 carbohydrate-binding region at a distant site).

This study, in conjunction with previous work, suggests that hemagglutination is mediated by a structural region of σ1 that is distinct from that mediating type-specific neutralization, tissue tropism, and L-cell binding. Anti-σ1 monoclonal antibodies were identified that were able to neutralize T3 strains but unable to inhibit HA; conversely, other anti-σ1 monoclonal antibodies were identified that were able to inhibit HA by T3 strains but unable to neutralize (6). These studies suggest that the functions of neutralization and HA are mediated by spatially separated regions of σ1. Variants of T3D that are resistant to neutralization by type-specific anti-σ1 monoclonal antibodies (26) have an altered pattern of central nervous system tropism in comparison with T3D (25). These variants have amino acid substitutions at residues 340 and 419 (4). Thus, sequences important for the binding of neutralizing monoclonal antibodies and for determining central nervous system tropism are widely separated from sequences identified in this study as important for HA and glycophorin binding (amino acids 198 to 204). Studies of σ1 deletion mutants expressed in *Escherichia coli* (16) and studies of protease digestion products of T3D σ1 (33) showed that sequences in the carboxy-terminal one-half of σ1 are sufficient to mediate L-cell binding but not HA. This suggests that σ1 sequences important for L-cell binding are also distinct from those important for HA.

Observations made in this study suggest that residues important for HA and glycophorin binding reside in a discrete region of the σ1 tail. Binding to carbohydrate ligands is important for reovirus binding to L cells and to erythrocytes (1, 11, 12, 19, 20) and is likely to be mediated by the region of sequence within the σ1 tail that we have identified as important for HA and glycophorin binding. However, in

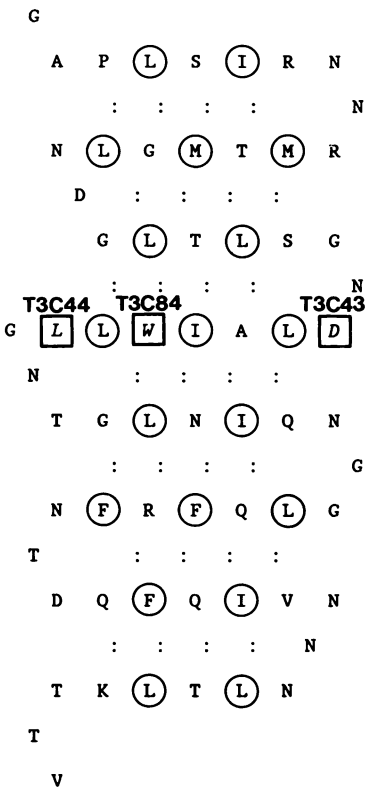

FIG. 3. Morphologic region T(iii) contains residues proposed to be important for hemagglutination. Single unique residues in HA-negative strains reside in a region of sequence (amino acids 174 to 237) in which conserved apolar residues conform to an alternating polar-apolar repeat pattern. A region of β-sheet secondary structure that is formed from a series of short β-strands separated by β-turns is depicted for this region of the T3D σ1 amino acid sequence as a series of short lines connected by turns (17). Repeat positions occupied by apolar residues in the proposed β-strands are circled. Hydrogen bonds proposed to occur between β-strands (:) are indicated. Residues unique to HA-negative strains are substituted in the T3D sequence and indicated in boxes (aspartate 198, T3C43; tryptophan 202, T3C84; and leucine 204, T3C44).

other studies, a region of sequence identified as sufficient for L-cell binding (16, 33) is predominantly contained within the σ1 head (17). Thus, reovirus attachment to cells may involve sequences in the σ1 tail which mediate binding to carbohydrates and sequences in the σ1 head which mediate binding to L-cell receptor proteins. Both of these regions may be required for the formation of a high-affinity virus-cell complex. Alternatively, these regions may function sequentially to mediate reovirus attachment to cells; the initial event in reovirus cell attachment may involve low-affinity binding to carbohydrate ligands by sequences in the σ1 tail, followed by high-affinity binding to protein receptors by sequences in the σ1 head. It is also possible that these binding activities may have different functional importance in reovirus infection of different cells and tissues.

The identification of a σ1 region important for HA suggests a model for attachment of σ1 to virions. Bassel-Duby et al. (3) observed that the amino-terminal one-third of the deduced T3D σ1 amino acid sequence consists of a heptad repeat pattern of apolar residues. This sequence motif is consistent with the formation of a fibrous tail constructed from an α-helical coiled-coil structure. These observations led to the proposal that σ1 is attached to virions by interac-

tions between the tail-forming sequences of σ1 and a central channel of the λ2 core spikes (3). However, in electron micrographs of virion particles of T2 Jones, Furlong et al. (10) observed σ1 to be extended from the surface of virions and concluded that only a small portion of the σ1 tail is attached to the virion when σ1 is in the extended conformation. In order for σ1 to assume this conformation, Furlong et al. (10) suggested that the σ1 tail must either be extruded from the λ2 channels or must not be folded entirely within these channels but folded at the virion surface instead. Our finding of a region important for HA within the σ1 tail supports the hypothesis that the σ1 tail is folded on the virion surface. In contrast to the model for σ1 attachment to virions proposed by Bassel-Duby et al. (3), our findings suggest that the σ1 tail is, in part, exposed to the virion surface, where amino acid residues important for HA and glycophorin binding can interact with carbohydrate ligands on cell surfaces.

We express our appreciation to Elaine Freimont and Mary Anne Mann for expert technical assistance. We are grateful to Deirdre Furlong for advice in constructing models of σ1. We thank Kenneth Tyler and Herbert Virgin for sharing their glycophorin-binding technique and preliminary results before publication. We also thank Kevin Coombs, Lynda Morrison, Leslie Schiff, and Greame Wilson for essential discussions and reviews of the manuscript.

This work was supported by Public Health Service research grant 5R37 AI13178-13 from the National Institute of Allergy and Infectious Diseases. Support to B.N.F. was also provided by the Shipley Institute of Medicine. M.L.N. was supported by National Research Service Award 5T32 GM07753-10 from the National Institute of General Medical Sciences. T.S.D. was the recipient of Physician-Scientist Award 5K11 AI00865 from the National Institute of Allergy and Infectious Diseases.

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