

JUNCTIONAL ADHESION MOLECULE-A AND
REOVIRUS PATHOGENESIS

By

Annukka Aida Rose Antar

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Approved:

Professor H. Earl Ruley

Professor Mark R. Denison

Professor Ethan Lee

Professor Terence S. Dermody

Professor Christopher R. Aiken

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To my parents, who raised me to love, wonder, and explore

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CHAPTER I

BACKGROUND

Introduction

Several unrelated viruses, including adenovirus, herpes simplex virus, poliovirus, reovirus, and rhinovirus, utilize receptors that are members of the immunoglobulin superfamily (IgSF) (4, 10, 49, 51, 82). IgSF members are heterogeneous in tissue distribution and biological function, but all share a common structural motif: the Ig fold. The Ig fold is a sandwich of two β sheets whose component β -strands are connected with a distinct topology (55). Members of the IgSF are commonly associated with functions in the immune system and include antibodies, antigen receptors, CD4, CD8, and class I and II major histocompatibility complex (MHC) proteins. Several IgSF virus receptors, including the reovirus receptor junctional adhesion molecule-A (JAM-A), are located in either tight junctions (TJs) or adherens junctions of polarized epithelial and endothelial cells (28, 80, 126, 127). Why viruses would utilize IgSF receptors located in cell-cell junctions is largely an unanswered question. In my dissertation research, I have attempted to define the role of JAM-A in reovirus pathogenesis. A detailed understanding of how reovirus interactions with JAM-A mediate host infection may illuminate principles of pathogenesis common to the viruses that bind junctional IgSF receptors.

Mammalian orthoreoviruses (reoviruses) form nonenveloped, double-shelled particles containing a genome of 10 segments of double-stranded (ds) RNA (Figure I-1)

(114). The prototype strains of reovirus were isolated in the 1950s (103, 111). Since that time, studies with reovirus have yielded several important discoveries in virology. For example, one of the first virology studies that employed genetic techniques identified allelic traits that segregated with particular reovirus gene segments (117). Building on these studies, it was first demonstrated with reovirus that a viral receptor attachment protein can mediate viral tropism and virulence (149). For nearly half a century, reovirus has proven to be a highly tractable experimental model for studies of viral pathogenesis (143).

When this work was initiated, JAM-A had been identified as a reovirus receptor, but it was unknown how JAM-A mediates reovirus infection in vivo. In Chapter II, I report that JAM-A is required for systemic infection of mice by reovirus. In Chapter III, I present work demonstrating that JAM-A is dispensable for reovirus neurotropism but required for promoting dissemination of reovirus within the infected host. In Chapter IV, I report that JAM-A mediates establishment of reovirus viremia and efficient infection of endothelial cells. These findings demonstrate that JAM-A is required for systemic dissemination of reovirus and suggest a role for junction-associated viral receptors in viremic dissemination.

Reovirus tropism and pathogenesis

Reoviruses have a wide geographic distribution, and virtually all mammals, including humans, serve as hosts for infection (134). However, reovirus is rarely associated with disease, except in the very young (76, 128). Reovirus strains can be

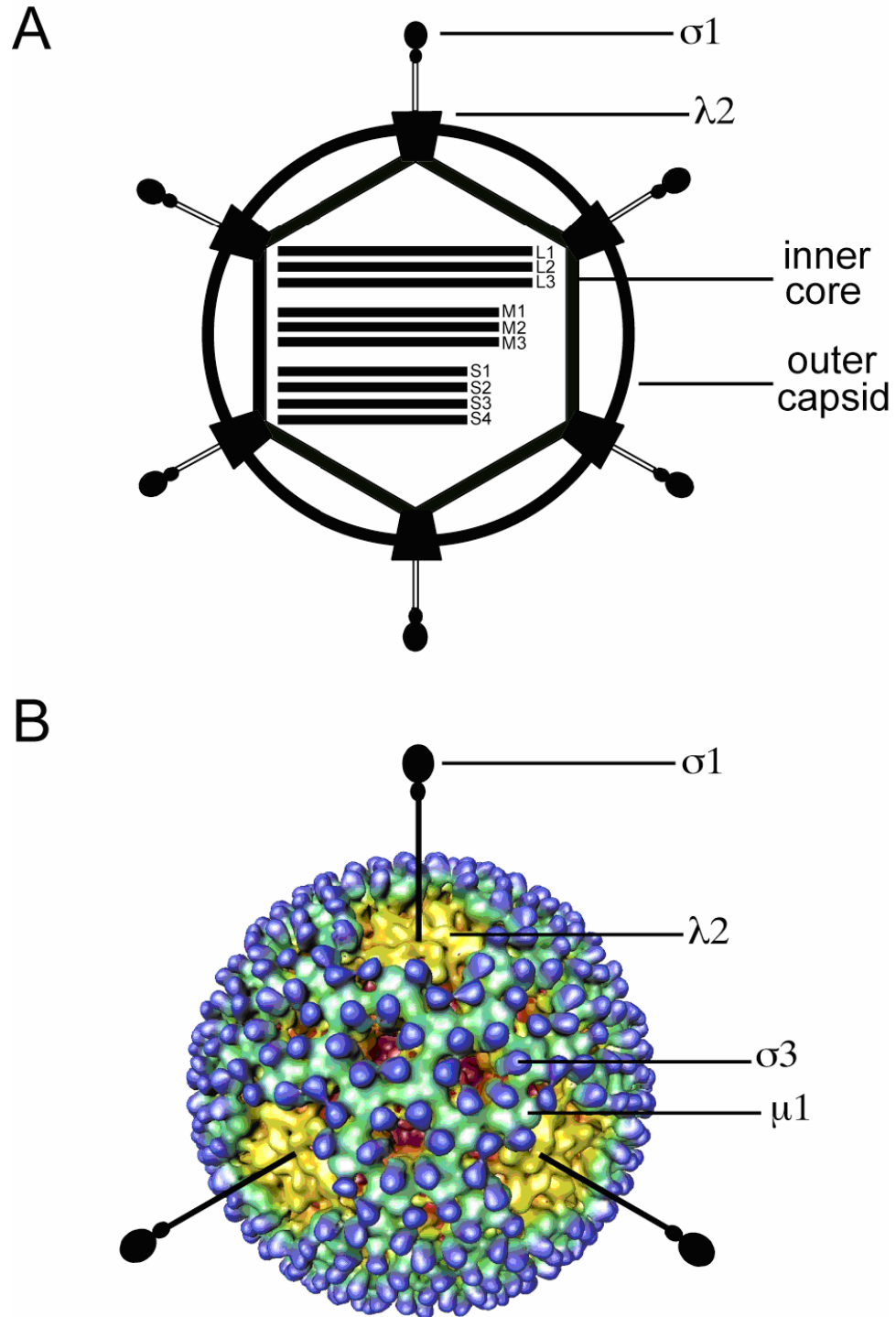


FIGURE I-1. A reovirus virion. (A) Schematic representation of a reovirus virion depicting 10 gene segments, a double-layered viral capsid, and the receptor attachment protein $\sigma 1$ inserted into pentamers of the structural protein $\lambda 2$. (B) Cryoelectron microscopic image reconstruction of a reovirus virion. Major outer capsid proteins are pseudocolored and labeled. $\sigma 1$ molecules are drawn schematically. Image adapted from Nason et al (89).

classified into three distinct serotypes based on neutralization and hemagglutination-inhibition tests (107, 111, 122). The prototype strain for reovirus type 1, type 1 Lang (T1L), was isolated from a healthy child. The prototype strains for reovirus types 2 and 3, type 2 Jones (T2J) and type 3 Dearing (T3D), respectively, were isolated from children with diarrhea (103, 111). Approximately 50% of healthy children acquire antibodies to T3D by the age of 5 years (125).

The natural portal of entry for reoviruses into the host is from the respiratory and enteric tracts (111). Newborn mice are exquisitely sensitive to reovirus infection and are the preferred experimental system for studies of reovirus pathogenesis (143). Infection of newborn mice by T1 and T3 reovirus strains via the enteric tract has been extensively studied (94). Following peroral inoculation, the reovirus outer capsid is processed by host intestinal proteases, resulting in the transition from the intact virion to a disassembly intermediate known as the infectious subvirion particle (ISVP) (13). Reovirus ISVPs specifically adhere to intestinal microfold (M) cells (Figure I-2), which overlie submucosal collections of lymphoid tissue called Peyer's patches (1, 6, 155). M cells are unique epithelial cells specialized for transepithelial transport of microbes and antigen (68). Virus is transported across M cells within endocytic vesicles and delivered to the basolateral surface (153-155). Reovirus antigen is then observed in mononuclear cells and dendritic cells of intestinal Peyer's patches (45, 61, 85, 155). By 48 hours postinoculation, reovirus antigen is observed in epithelial cells of the ileum (109). Reovirus T1L and T3D differ in the capacity to infect the intestine following peroral inoculation, a property that segregates with the viral S1 and L2 genes (12). This

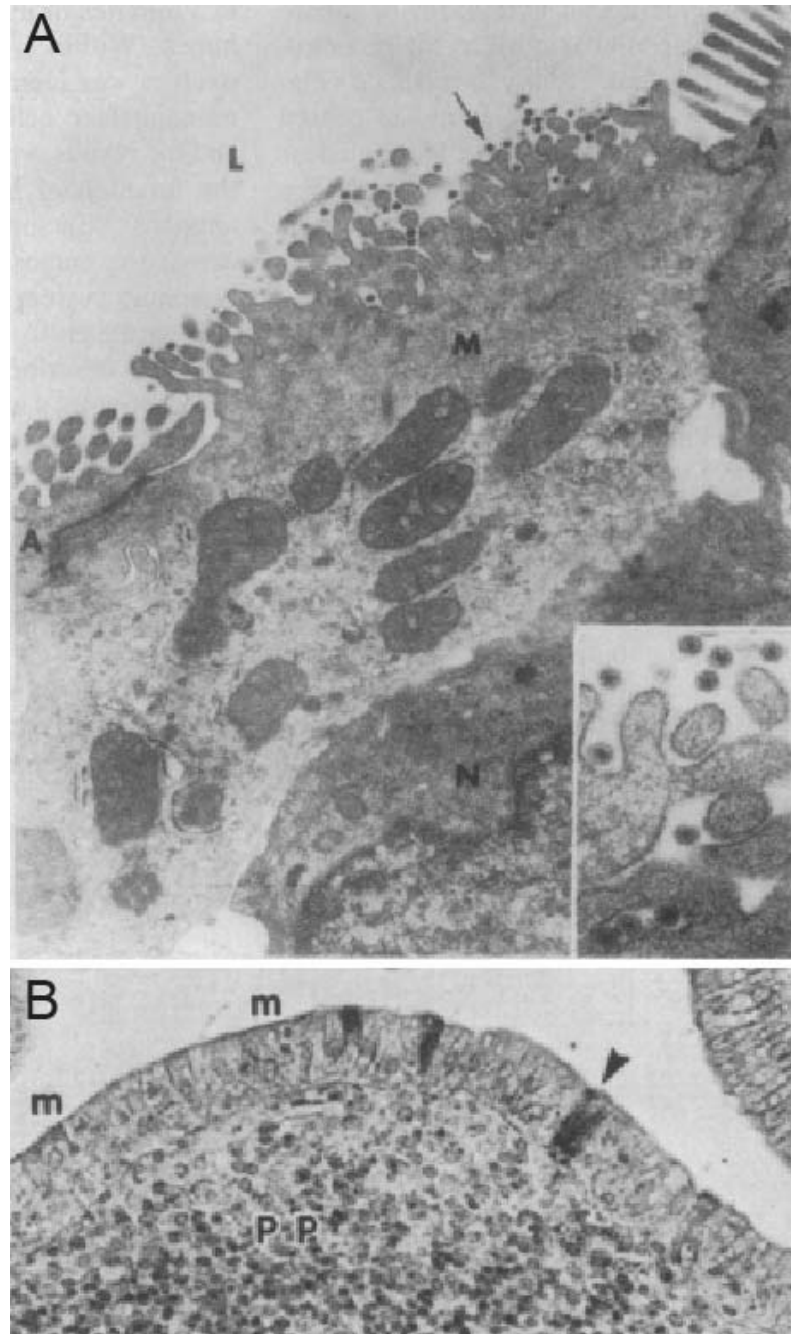


FIGURE I-2. Reovirus T1L binds and infects M cells that overlie Peyer's patches. (A) An electron micrograph demonstrating T1L virions (arrow) adhering to the luminal (L) surface of an M cell (M) in a closed ileal loop 30 minutes after virus inoculation. The M cell surrounds a mononuclear cell (N) and borders two absorptive (A) cells that lack adherent virus (x13,400). Inset: higher magnification of M cell surface with adherent virions (x38,800). (B) A photomicrograph of adult mouse intestine 48 h after inoculation with 5×10^9 PFU T1L demonstrates reovirus antigen-positive (arrowhead) M cells (m) overlying Peyer's patches (PP). Images adapted from Wolf et al (155) (A) and Rubin et al (109) (B).

difference in intestinal infection is due to proteolytic cleavage of the T3D $\sigma 1$ attachment protein, which is encoded by the S1 gene (23). I helped to demonstrate that a T3D virus containing a mutation that abolishes this cleavage site in $\sigma 1$ replicates in the intestine to higher titer than T3D (Appendix B, 66).

Both T1 and T3 strains disseminate systemically and invade the central nervous system (CNS) following peroral inoculation of newborn mice, but they do so via different modes of spread and produce distinct pathologic consequences. Reovirus T1 spreads from the intestine to the mesenteric lymph node and spleen within 24 hours of inoculation, presumably via hematogenous routes (61). In contrast, reovirus T3 clone 9 spreads from the intestine to the myenteric plexus to the dorsal motor nucleus of the vagus nerve and thus uses a neural route to spread from the intestine to the CNS (85). Once in the CNS, T1 reovirus infects ependymal cells (136, 149), resulting in hydrocephalus (148). T3 reovirus exhibits strikingly different tropism within the CNS, infecting neurons in the cortex, thalamus, and hippocampus (Figure I-3) (85, 136, 149) and causing lethal encephalitis (128, 148).

The serotype-determining S1 gene of reovirus mediates differences between T1 and T3 strains in mode of spread, CNS tropism, and neurovirulence (135, 136, 148, 149). S1 gene product $\sigma 1$ is a filamentous, trimeric molecule that extends from the virion surface (48) and mediates viral attachment to host-cell receptors (4, 71, 147). In addition to $\sigma 1$, the S1 gene also encodes a small nonstructural protein, $\sigma 1s$ in an overlapping reading frame. The $\sigma 1s$ protein is not required for growth in cell culture but is thought to mediate reovirus-induced G(2)/M cell-cycle arrest (42, 59, 99, 106, 113, and K. W. Boehme and T. S. Dermody, manuscript in preparation).

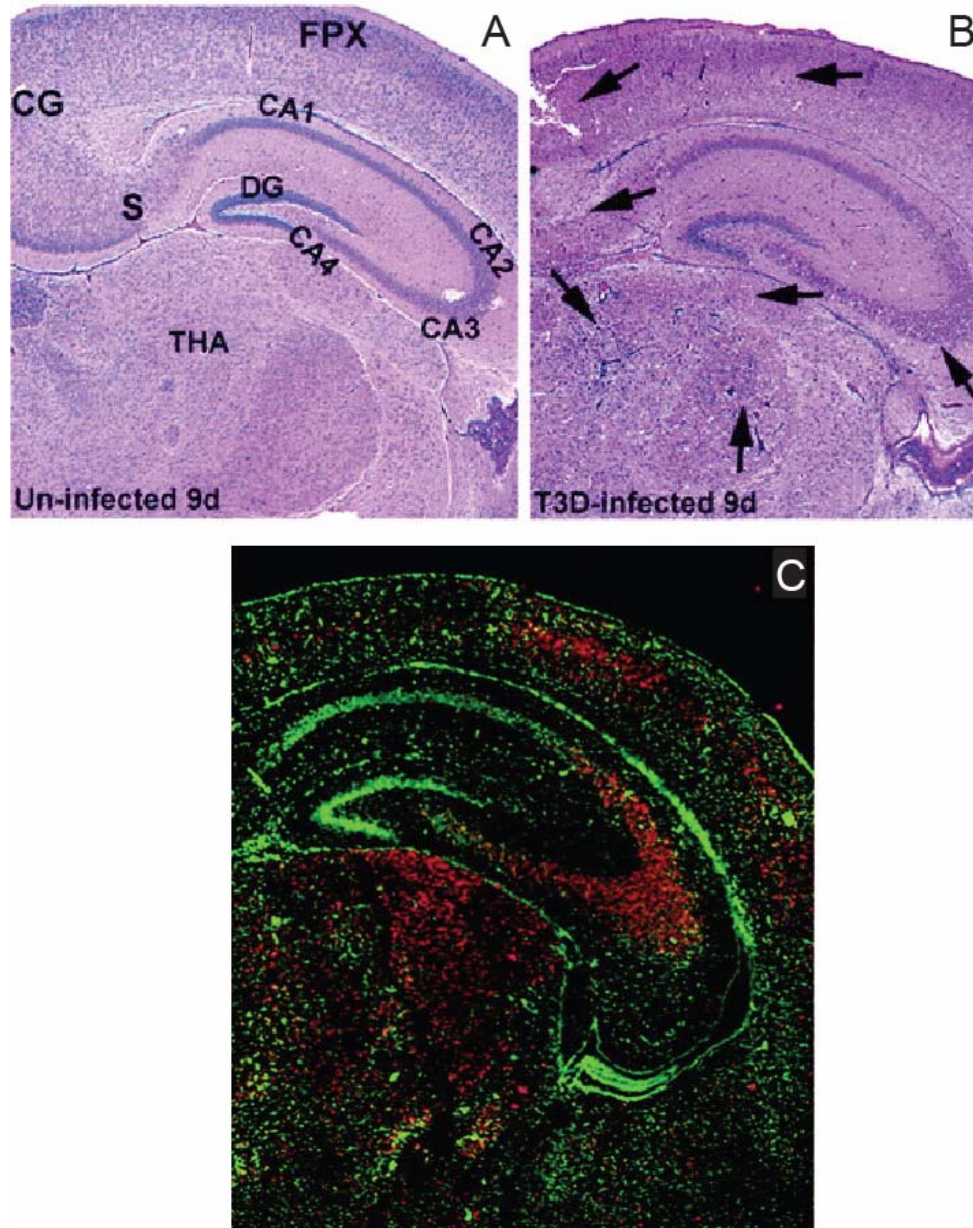


FIGURE I-3. T3 reovirus tropism in the brain. (A) A coronal brain section from an uninoculated 9-day-old mouse pup exhibits normal cellular architecture with hematoxylin and eosin (H&E) stain. CG, cingulate gyrus; FPX, frontoparietal cortex; S, subiculum; DG, dentate gyrus; THA, thalamic nuclei; CA1-4, hippocampal regions. (B) A coronal brain section from a 9-day-old mouse pup 7 days following intracranial inoculation with T3D demonstrates abnormal cytoarchitecture, massive cell loss, and minor inflammation throughout the cortical, hippocampal, and thalamic regions. Arrows point to areas of severe viral injury. (C) A coronal brain section from a 9-day-old mouse pup 7 days following intracranial inoculation with T3D stained for reovirus antigen (red) shows strong positive staining throughout the cortical, hippocampal, and thalamic regions of the brain. Neurons are counterstained with fluorescent Nissl stain (green cells). Images adapted from Richardson-Burns et al (105).

Attachment and internalization of reovirus

The first step in a viral infectious cycle is the engagement of receptors on the surface of host cells. Findings from several different virus families indicate that viral attachment to cells often involves multiple interactions between several viral and cellular molecules (33). The reovirus attachment protein $\sigma 1$ forms an elongated fiber with a compact globular head at the C-terminus (26, 47) and binds JAM-A (4) and carbohydrate. The $\sigma 1$ head mediates JAM-A binding, whereas residues in the $\sigma 1$ tail, close to the midpoint of the molecule, mediate T3 $\sigma 1$ binding to sialic acid (23, 24). A region in the tail just beneath the head mediates T1 $\sigma 1$ binding to a carbohydrate that has not been definitively identified (24, 57).

The contribution of sialic acid to cellular attachment and host virulence has been studied using monoreassortant reoviruses containing the $\sigma 1$ -encoding S1 gene of either nonsialic acid-binding strain T3C44 (T3SA-) or sialic acid-binding strain T3C44-MA (T3SA+) (3, 5). The remaining nine genes of T3SA+ and T3SA- derive from T1L. These virus strains are capable of growth in the intestine because their $\sigma 1$ does not share cleavage susceptibility with the T3D $\sigma 1$ (23). T3SA+ and T3SA- differ in sialic acid-binding capability by virtue of a single substitution (Leu²⁰⁴ to Pro) in the $\sigma 1$ tail (25). Studies using these virus strains indicate that multiple, low-affinity reovirus-sialic acid interactions enhance initial adhesion of the virus to the cell surface and facilitate later, higher affinity binding to JAM-A (3). Pathogenesis studies using these strains suggest that the capacity to bind sialic acid enhances the kinetics of dissemination, increases yields in target organs, confers tropism to bile duct epithelial cells, and mediates an obstructive liver disease in mice similar to human infantile biliary atresia (5).

The entry and disassembly of reovirus in a cell is a complex, yet coordinated, program and requires several viral outer-capsid components and host cell molecules. After engaging receptors, reovirus is internalized into cells via a mechanism dependent on $\beta 1$ integrin (72). The $\beta 1$ integrin NPXY motifs, which are potential sites of tyrosine phosphorylation and serve as endocytic sorting signals (14), are required for functional reovirus entry (73). Studies suggest that reovirus is internalized via clathrin-dependent endocytosis (15, 16, 41, 110, 124). Reovirus subsequently undergoes stepwise disassembly within minutes of internalization that is dependent on acidic pH (77, 124) and endosomal cysteine-containing proteases cathepsins B and L (39). This disassembly generates well-defined intermediates. The ISVP lacks the outer-capsid protein $\sigma 3$ and contains two new particle-associated peptides, δ and φ , generated by cleavage of the outer-capsid protein $\mu 1$ (16, 22, 119, 124). ISVPs are further processed to ISVP*s by loss of $\sigma 1$ and conformational rearrangement of δ and φ . The ISVP* penetrates endosomes to deliver transcriptionally active viral cores into the cytoplasm (20, 21, 92, 93).

JAM-A is a reovirus receptor

Evidence accumulated in the past twenty years had indicated that the $\sigma 1$ head promotes receptor interactions that are distinct from interactions with sialic acid mediated by the $\sigma 1$ tail (7, 24, 25, 37, 38, 62, 91, 120, 121, 131). Our laboratory employed a flow cytometry-based expression-cloning approach to identify a receptor bound by the reovirus $\sigma 1$ head (4). T3SA- was used as the affinity ligand to avoid isolating glycosylated molecules that might not interact specifically with $\sigma 1$. A cDNA library

generated from NT2 cells, a human testicular embryonal carcinoma cell line with epithelial-like properties in culture, was selectively enriched for cDNAs that confer binding of fluoresceinated virions to transfected cells. Four clones conferred virus binding; each encoded human JAM-A (hJAM-A). hJAM-A-specific monoclonal antibodies were found to inhibit T1 and T3 reovirus binding and infection. Expression of hJAM-A and murine JAM-A (mJAM-A) in nonpermissive cells permitted T1 and T3 reovirus growth. The $\sigma 1$ protein was demonstrated to bind directly to hJAM-A with an apparent K_D of approximately 6×10^{-8} M (4). Together, these findings indicate that both hJAM-A and mJAM-A serve as receptors for T1 and T3 reovirus. Later work demonstrated that hJAM-A, but not the related proteins hJAM-B, hJAM-C, or human coxsackievirus and adenovirus receptor (hCAR), serves as a receptor for both prototype and field-isolate strains of all three reovirus serotypes (18, 101). Currently, JAM-A is the only known proteinaceous receptor for reovirus.

Interactions of JAM-A and $\sigma 1$

Since the identification of JAM-A as a reovirus receptor, many structural and functional details of its interaction with $\sigma 1$ have been elucidated. A surface at the base of the $\sigma 1$ head domain predicted to bind JAM-A was identified by mapping $\sigma 1$ residues conserved among the three serotypes onto the crystal structure of $\sigma 1$ (18, 26). The crystal structure of the extracellular region of hJAM-A revealed two Ig-type domains with a pronounced bend at the domain interface (Figure I-4 A). Two hJAM-A molecules form a dimer via their membrane distal V-type Ig domains (D1 domains) similar to that observed in the mJAM-A crystal structure (Figure I-4 B) (67, 101). Chimeric and domain deletion

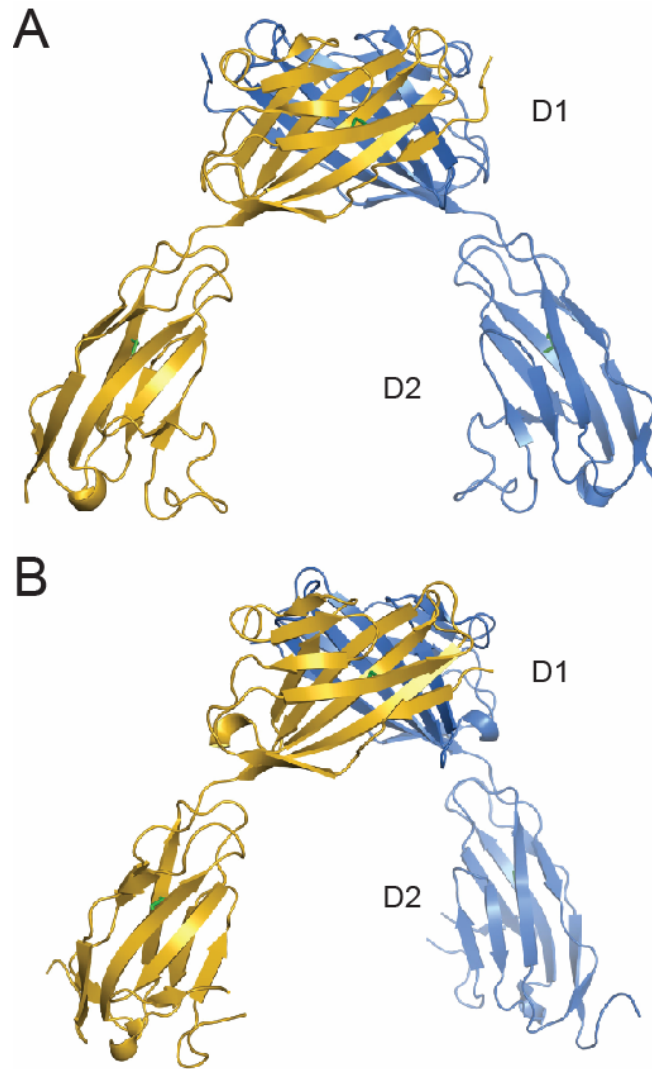


FIGURE I-4. Crystal structures of the hJAM-A and mJAM-A extracellular domains. (A-B) Ribbon drawings of the hJAM-A dimer (A) and the mJAM-A dimer (B), with one monomer shown in orange and the other in blue. Disulfide bonds are shown in green. Dimerization is mediated by the membrane-distal D1 immunoglobulin domain. Both human and murine JAM-A serve as reovirus receptors. Images adapted from Prota et al (101) (A) and Kostrewa et al (67) (B) by Eva Kirchner.

mutants of hJAM-A were used to demonstrate that the D1 domain of hJAM-A is required for reovirus attachment and infection (46). A structure-guided mutational analysis of the JAM-A dimer interface revealed that T3D σ 1 binds to monomers of JAM-A via residues found mainly on beta-strands C and C' of the JAM-A dimer interface (52). Similar

analyses using T1L and T2J demonstrated that the residues of JAM-A that are required for binding to these strains are similar but not identical to those required for T3D binding (52). A crystal structure has been solved of the amino-terminal domain of JAM-A in complex with the T3D σ 1 head (E. Kirchner, K. M. Guglielmi, T. S. Dermody, and T. Stehle, manuscript in preparation). This structure elucidates contact sites in each molecule and reveals that each σ 1 head domain binds to one JAM-A monomer (Figure I-5) (E. Kirchner, K. M. Guglielmi, T. S. Dermody, and T. Stehle, manuscript in preparation).

Interactions between T3D σ 1 and JAM-A parallels other virus-receptor systems. One such close parallel is the adenovirus fiber-CAR interaction. Adenovirus and reovirus belong to different virus families and share few common properties, but both are nonenveloped and use an attachment receptor with head-and-tail morphology (50). The key structural features shared by σ 1 and fiber include defined regions of flexibility within the tail, in part composed of an unusual triple β -spiral motif, and head domains that are formed by an 8-stranded β -barrel with identical β -strand connectivity (26, 139, 140, 157). Each viral attachment protein engages its receptor, a cell-surface, two-Ig domain, dimeric host protein expressed at cellular junctions, by interacting with the dimer interface and its top loops (11, 140, and E. Kirchner, K. M. Guglielmi, T. S. Dermody, and T. Stehle, manuscript in preparation). Moreover, CAR and JAM-A are related members of the JAM/cortical thymocyte marker of *Xenopus* (CTX) family. The crystal structures of each virus in complex with its receptor indicate that although the viral attachment proteins engage their receptors using different binding sites, the viral protein-bound area of the

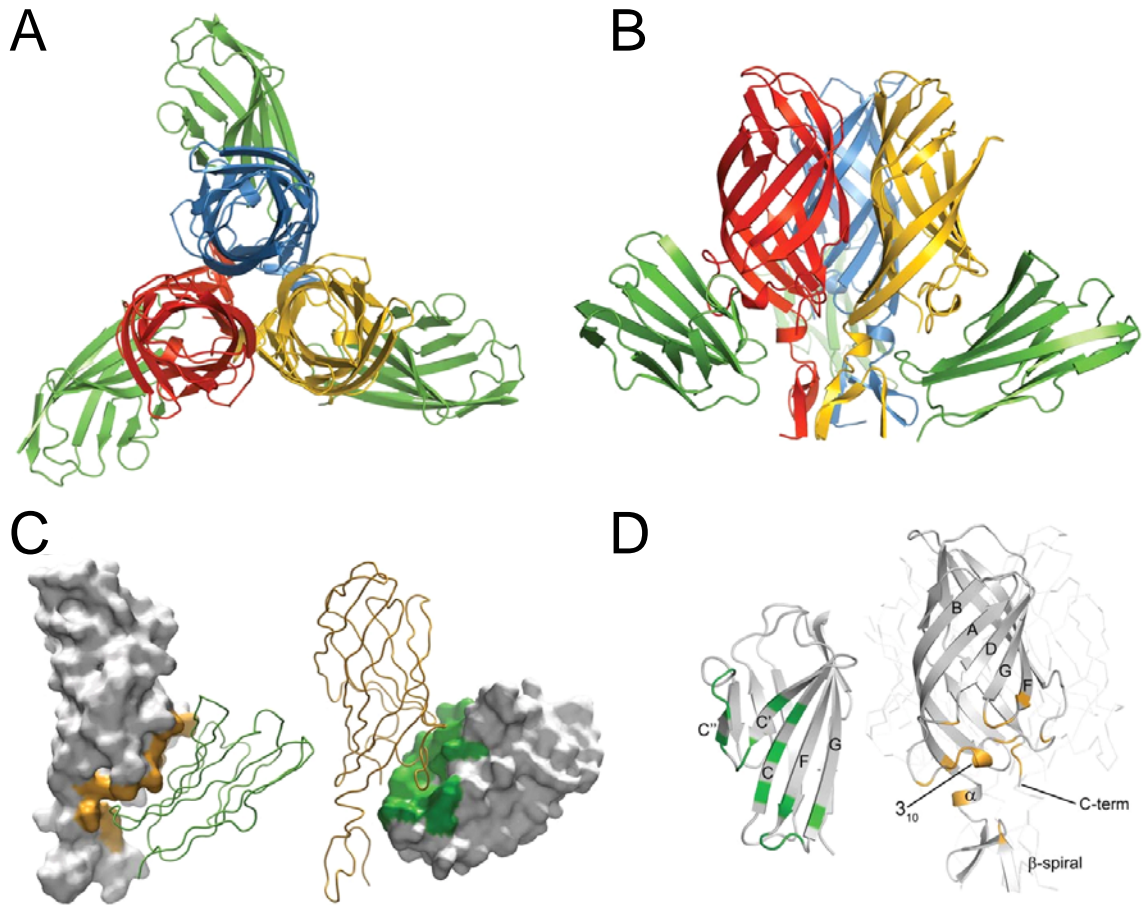


FIGURE I-5. Crystal structure of the T3D σ 1 head-JAM-A D1 complex. (A, B) Ribbon drawings of the complex between trimeric σ 1 head and monomeric JAM-A D1, viewed along the three-fold symmetry axis (A) and from the side (B). σ 1 head monomers are shown in blue, yellow, and red; D1 is shown in green. (C) Surface representation of the contact area of reovirus σ 1 head (left, orange) and D1 (right, green). Interacting partners are shown as ribbon traces. (D) Ribbon drawings of D1 (left) and σ 1 head (right). Secondary structure elements are labeled. Contact residues (distance cutoff 4 Å) in the σ 1 head-D1 interface are colored green (D1) or orange (σ 1H).

host receptor is similar (E. Kirchner, K. M. Guglielmi, T. S. Dermody, and T. Stehle, manuscript in preparation).

Roles of JAM-A in the host

The JAM family of proteins are IgSF members involved in epithelial barrier function, vascular permeability, and leukocyte transmigration (8, 146). In adult mice, JAM-A is expressed on epithelium and endothelium of a wide variety of organs and is also found on dendritic cells, lymphocytes, monocytes, macrophages, neutrophils, and platelets (8, 19, 80). JAM-A contains two extracellular Ig-like domains, a short transmembrane region, and a cytoplasmic tail possessing a PDZ-domain-binding motif (80, 152). The PDZ-binding motif facilitates the binding of JAM-A to TJ-associated cytoplasmic proteins such as zonula occludens-1 (ZO-1) (40), calcium/calmodulin-dependent serine protein kinase (CASK) (79), and multi-PDZ domain protein 1 (MUPP1) (56). JAM-A forms dimers and tetramers (67, 101).

JAM-A is an integral component of TJs (80), which form a barrier to intercellular passage of water and solutes (115). In this context, JAM-A plays a key role in leukocyte diapedesis in inflammation (146). Redistribution of JAM-A from cellular junctions to the apical surface occurs during an inflammatory response (96, 98) and is thought to allow JAM-A to engage circulating leukocytes for diapedesis through endothelium (118, 146). The interaction of JAMs with leukocyte integrins is thought to be important for immune cell diapedesis. Specifically, the membrane-proximal Ig domain (D2 domain) of endothelial JAM-A engages the leukocyte β_2 integrin LFA1 (97). Also, endothelial JAM-A can interact in *cis* with integrin $\alpha_v\beta_3$ (87, 88). JAM-A expression influences the expression of β_1 integrins (75), and expression of dimerization-defective JAM-A leads to internalization of β_1 integrin from the cell surface (116). In addition, dimerization of platelet and endothelial JAM-A in *trans* facilitates platelet adhesion to cytokine-

stimulated endothelium. Furthermore, JAM-A may be an evolutionary precursor to antigen-recognizing receptors and antigen-presenting molecules. Du Pasquier and colleagues point out that the JAM/CTX and nectin families of proteins are the most likely candidates found in urochordate and cephalochordate genomes to have duplicated and given rise to elements of the human adaptive immune system (36). Both of these protein families are notable for serving as virus receptors, and it has been proposed that interactions between viruses and these receptors may have driven the evolution of the adaptive immune system (36).

Despite the important functions of JAM-A in the host, *JAM-A*^{-/-} mice are viable and display no defects in organ development and morphology (19). In addition, these mice do not have altered numbers of circulating platelets, lymphocytes, neutrophils, or monocytes (19). However, the colonic mucosa of *JAM-A*^{-/-} mice demonstrates increased neutrophil infiltration, large lymphoid aggregates, and increased permeability, which is specific to epithelial cells (70). *JAM-A*^{-/-} mice also exhibit decreased leukocyte transmigration from blood to tissue in several models of inflammation and injury (30, 64, 156, 158). In addition, *JAM-A*^{-/-} mice demonstrate enhanced dendritic cell migration from skin to lymph node associated with increased contact hypersensitivity (19). A role for JAM-A in angiogenesis was highlighted by a study in which fibroblast growth factor-2-induced microvessel sproutings were inhibited in *JAM-A*^{-/-} mouse tissue (29).

JAM-A also interacts with microbes. JAM-A serves as a receptor for all three serotypes of reovirus (18) and feline calicivirus (74) and is also targeted by a bacterium. *Helicobacter pylori* translocates its CagA protein into epithelial cells, where it recruits ZO-1 and JAM-A from the TJ to adhere to bacteria and disrupts TJ-mediated gate and

fence functions (2). JAM-A thus plays key roles in the host by mediating TJ barrier function, inflammatory responses, angiogenesis, and interactions with pathogens.

Significance of the research

Little is known about how IgSF junction proteins function in the pathogenesis of the virus families that bind them. Therefore, elucidating the role of JAM-A in reovirus pathogenesis might contribute to a broader understanding of the contribution of similar receptors in the pathogenesis of their viral ligands. In addition, reovirus has been used in clinical trials for the treatment of aggressive human malignancies (123, 132). A precise understanding of how reovirus-JAM-A interactions affect viral tropism could contribute to improved design of viral vectors for oncolytic delivery purposes.

My work has focused on defining the role of JAM-A in reovirus pathogenesis. I found that JAM-A is responsible for reovirus dissemination via the blood but is neither required for viral replication in the intestine nor viral access to neural routes of spread. This work provides evidence for the existence of one or more additional reovirus receptors and enhances an understanding of systemic dissemination of virus families that employ junction-associated IgSF members as receptors.

CHAPTER II

JAM-A IS REQUIRED FOR EFFICIENT INFECTION OF CELLS AND SYSTEMIC INFECTION OF MICE BY REOVIRUS

Introduction

JAM-A is an IgSF member that serves as a receptor for feline calicivirus (74) and all three serotypes of mammalian reovirus (4, 18). Although JAM-A is the only known proteinaceous receptor for reovirus, nothing is known about the role of JAM-A in the pathogenesis of reovirus disease. In this study, I used wild-type and isogenic JAM-A-null mice to determine the extent to which JAM-A is required for reovirus infection of cells and animals. I found that JAM-A significantly enhances but is not absolutely required for infection of mouse embryonic fibroblasts (MEFs). Following peroral inoculation of newborn mice, I demonstrate that JAM-A is required for systemic reovirus infection and lethal reovirus-induced disease. These studies confirm that JAM-A is a primary cellular receptor for reovirus in cultured cells and reveal a new role for JAM-A in systemic infection of mice.

Results

T1 and T3 reoviruses efficiently infect wild-type but not JAM-A^{-/-} MEFs - To determine whether genetic deletion of JAM-A alters reovirus infection of cells, I generated primary MEFs from wild-type and isogenic *JAM-A^{-/-}* mice and infected those cells with T1 and T3 reovirus strains at various multiplicities of infection (MOIs). All strains of reovirus were capable of infecting wild-type MEFs but not *JAM-A^{-/-}* MEFs over the course of a single

infectious cycle (Figure II-1 A and B). I tested the efficiency of viral replication over several days and found that JAM-A is required for efficient reovirus replication in MEFs (Figure II-1 C). Strains T3D and T3SA+, which bind sialic acid (3, 32) replicated to higher titer in *JAM-A*^{-/-} MEFs than did reovirus strains T1L and T3SA-, which do not bind this carbohydrate (3), suggesting that the capacity to bind sialic acid enhances reovirus infection of MEFs. Taken together, these results demonstrate that JAM-A expression is required for efficient infection of MEFs by T1 and T3 reoviruses.

T3D and T3SA+ reovirus infection of JAM-A^{-/-} *MEFs is reduced following neuraminidase treatment* - To determine whether sialic acid binding mediates residual replication of sialic acid-binding reovirus strains in *JAM-A*^{-/-} MEFs, I quantified viral replication following incubation of cells with a mock treatment or with *Arthrobacter ureafaciens* neuraminidase to remove cell-surface sialic acid. T3D and T3SA+ infection of both wild-type and *JAM-A*^{-/-} MEFs was reduced following treatment of cells with neuraminidase (Figure II-2). Consistent with previous findings using HeLa cells (3), neuraminidase treatment did not decrease infection of wild-type or *JAM-A*^{-/-} MEFs with T3SA- (Figure II-2). These data indicate that reovirus T3D and T3SA+ replication in *JAM-A*^{-/-} MEFs is in part mediated by sialic acid.

T3 reovirus replication in *JAM-A*^{-/-} MEFs may also be mediated by a receptor other than JAM-A or sialic acid. To determine whether T3 reovirus replication in *JAM-A*^{-/-} MEFs depends on σ 1 head-specific interactions, I quantified viral replication following incubation of virus and cells with the T3 σ 1 head-specific blocking monoclonal antibody (MAb) 9BG5 (17). Treatment with MAb 9BG5 substantially reduced infectivity of T3D, T3SA+, and

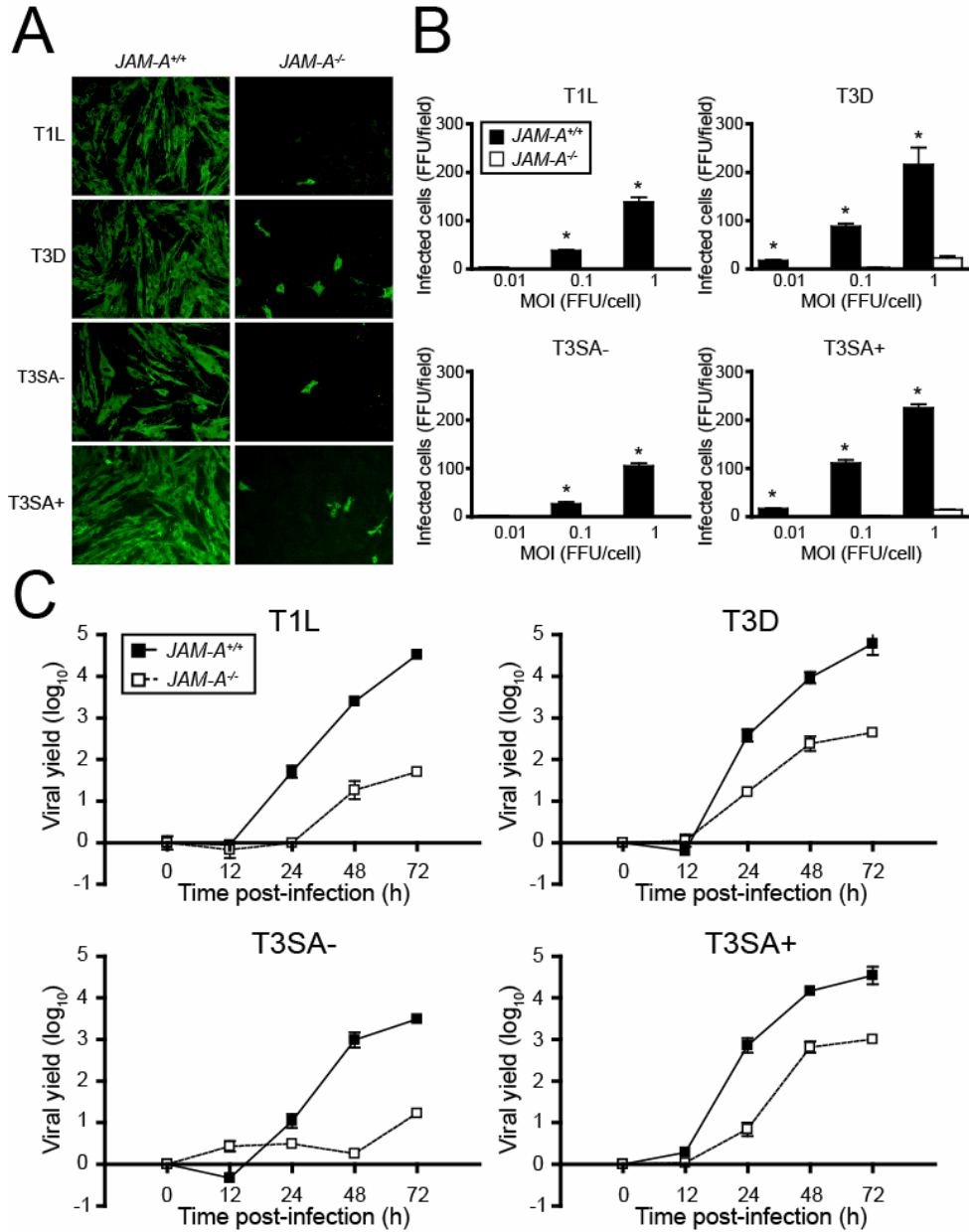


FIGURE II-1. JAM-A is required for efficient reovirus infection of MEFs. (A) Primary MEFs generated from *JAM-A*^{+/+} and *JAM-A*^{-/-} embryos were adsorbed with reovirus T1L, T3D, T3SA-, or T3SA+ at MOIs of 0.01, 0.1, and 1 fluorescent focus unit (FFU)/cell and incubated for 20 hr. Reovirus antigen was detected by indirect immunofluorescence. Representative wells after adsorption with 1 FFU/cell are shown. (B) Infected cells were quantified in five fields of 200X view. Results are expressed as the mean FFU/field for triplicate experiments. Error bars indicate SD. *, *P* < 0.01 as determined by Student's *t* test in comparison to *JAM-A*^{-/-} MEFs at the same MOI. (C) Confluent monolayers of *JAM-A*^{+/+} and *JAM-A*^{-/-} MEFs were adsorbed with reovirus T1L, T3D, T3SA-, or T3SA+ at an MOI of 2 plaque forming units (PFU)/cell and incubated for the times shown. Viral titers were determined by plaque assay. Results are expressed as mean viral yields (*t_x/t₀*) for triplicate experiments. Error bars indicate SD.

T3SA- in wild-type MEFs (Figure II-2). Infection of *JAM-A*^{-/-} MEFs with T3SA+ was reduced by one third with 9BG5 treatment compared to the mock-treated control (Figure II-2). However, infection of *JAM-A*^{-/-} MEFs with T3D and T3SA- was not altered following 9BG5 treatment. Residual T3 reovirus infection of *JAM-A*^{-/-} MEFs is therefore likely not mediated by T3 σ 1 head-specific interactions.

JAM-A is required for lethal reovirus infection following peroral inoculation - To determine the function of JAM-A in reovirus pathogenesis, I compared the susceptibility of wild-type and *JAM-A*^{-/-} mice to reovirus infection and disease. I isolated the role of JAM-A from the role of sialic acid in reovirus pathogenesis by using T3SA-, a reovirus strain incapable of binding sialic acid (3). T3SA- spreads systemically and retains T3 neurotropic properties (5). Newborn wild-type and *JAM-A*^{-/-} mice were inoculated perorally with escalating doses of T3SA-, from 10 to 10⁹ plaque forming units (PFU), and survival was assessed as a function of dose. The percentage of wild-type mice that succumbed to lethal encephalitis rose with increasing dose, yielding an LD₅₀ value of 1.9 x 10⁷ PFU (Table II-1). In sharp contrast, no *JAM-A*^{-/-} animals succumbed to lethal encephalitis following inoculation with T3SA- at any dose tested (Table II-1), nor did *JAM-A*^{-/-} mice demonstrate detectable neurological symptoms. At the highest dose of T3SA- tested, 10⁹ PFU, the majority of wild-type mice succumbed to lethal encephalitis, whereas all *JAM-A*^{-/-} mice survived (Figure II-3 A). As a quantitative indicator of disease progression, I found that surviving wild-type mice inoculated with 10⁹ PFU gained weight less rapidly than similarly inoculated *JAM-A*^{-/-} mice (Figure II-3 B). Taken

together, these results demonstrate that JAM-A is required for lethal reovirus disease following peroral inoculation of T3SA-.

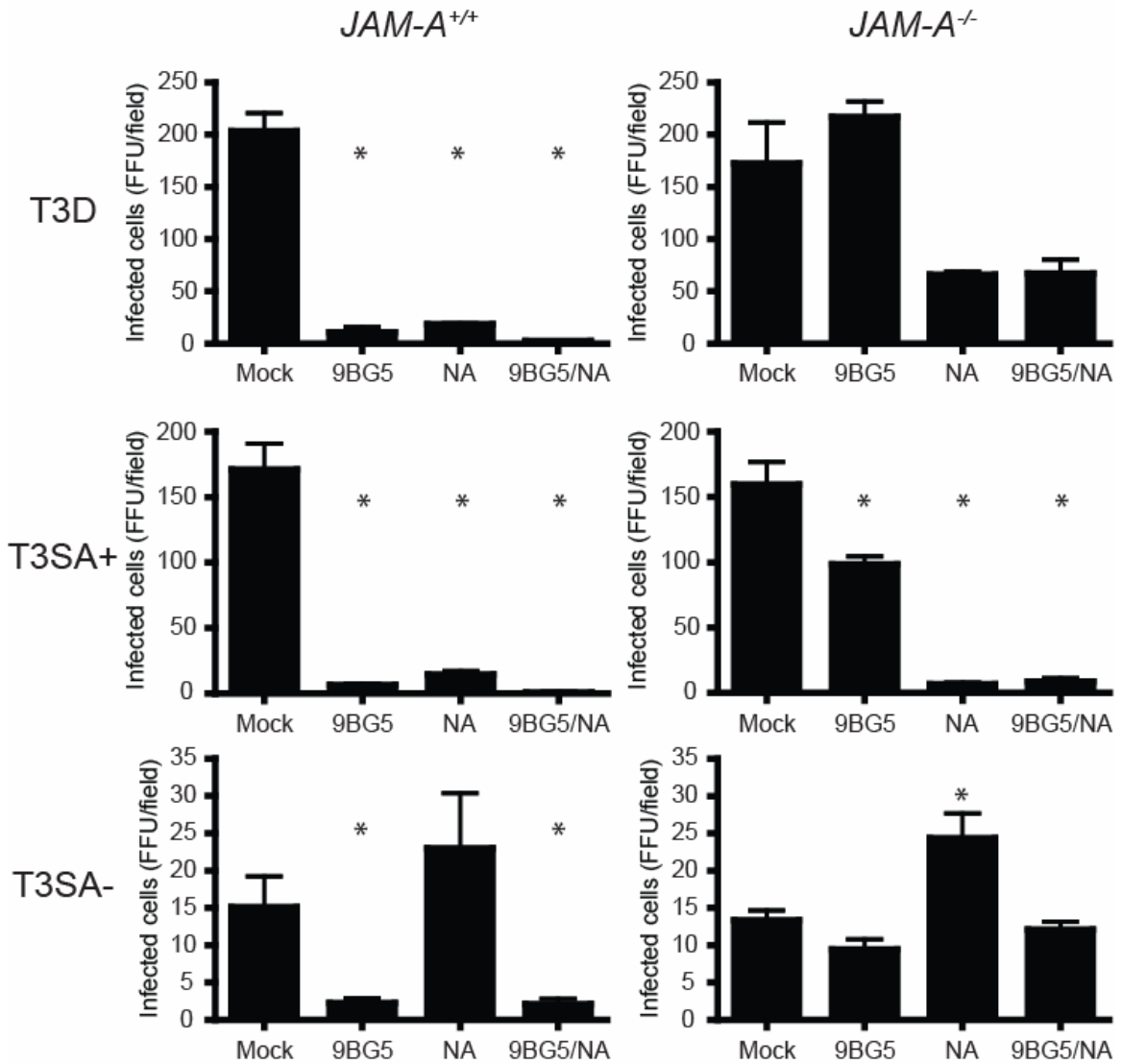


FIGURE II-2. Neuraminidase treatment diminishes T3 infection of *JAM-A*^{-/-} MEFs. Primary MEFs generated from *JAM-A*^{+/+} and *JAM-A*^{-/-} embryos were treated with incomplete media or 100 mU/mL neuraminidase. Cells were adsorbed with reovirus T3D, T3SA+, or T3SA- at MOIs of 4×10^4 particles/cell (*JAM-A*^{+/+}) or 4×10^6 particles/cell (*JAM-A*^{-/-}) combined with incomplete medium or with 20 μ g/mL 9BG5 and incubated for 20 hr. Reovirus antigen was detected by indirect immunofluorescence. Infected cells were quantified in five fields of 200X view (*JAM-A*^{+/+}) or five fields of 160X view (*JAM-A*^{-/-}). Results are expressed as the mean FFU/field for triplicate experiments. Error bars indicate SD. *, $P < 0.05$ as determined by Student's *t* test in comparison to mock treated MEFs.

TABLE II-1. JAM-A is required for T3SA- virulence following peroral inoculation^a.

Dose (PFU/mouse)	<i>JAM-A</i> ^{+/+}		<i>JAM-A</i> ^{-/-}	
	Survive	Succumb	Survive	Succumb
10	20	0	11	0
10 ²	13	0		
10 ³	14	0	15	0
10 ⁴	5	10		
10 ⁵	16	4	15	0
10 ⁶	9	5		
10 ⁷	13	1	14	0
10 ⁸	6	5		
10 ⁹	6	20	16	0

^aNewborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with the indicated doses of T3SA-. Each dose was used to inoculate 2-3 litters of mice. Animals were monitored for survival for 21 days after inoculation, at which point survivors appeared healthy. LD₅₀ values were calculated according to the method of Reed and Muench (104). The LD₅₀ value of T3SA- in *JAM-A*^{+/+} mice is 1.9 x 10⁷ PFU. The LD₅₀ value of T3SA- in *JAM-A*^{-/-} mice is > 10⁹ PFU.

JAM-A is required for systemic reovirus replication following peroral inoculation of T3SA- - Although peroral inoculation of T3SA- does not cause reovirus-induced disease in *JAM-A*^{-/-} mice, I wondered whether reovirus is capable of replication in *JAM-A*^{-/-} animal tissues. To determine the extent of viral replication in infected mice, I inoculated newborn wild-type and *JAM-A*^{-/-} mice perorally with 10⁴ PFU T3SA-, a sub-LD₅₀ value, and quantified viral titer in organs at various intervals post-inoculation. It is known that titers in the intestine are negligible at days 4 and 8 following peroral inoculation of reovirus strains that do not replicate in the intestine, such as T3D (12). Therefore, viral titer in the intestine at and after 4 days post-inoculation is indicative of reovirus replication at that site. Remarkably, T3SA- produced equivalent titers of virus in the intestine of wild-type and *JAM-A*^{-/-} mice. However, viral titers at sites of secondary

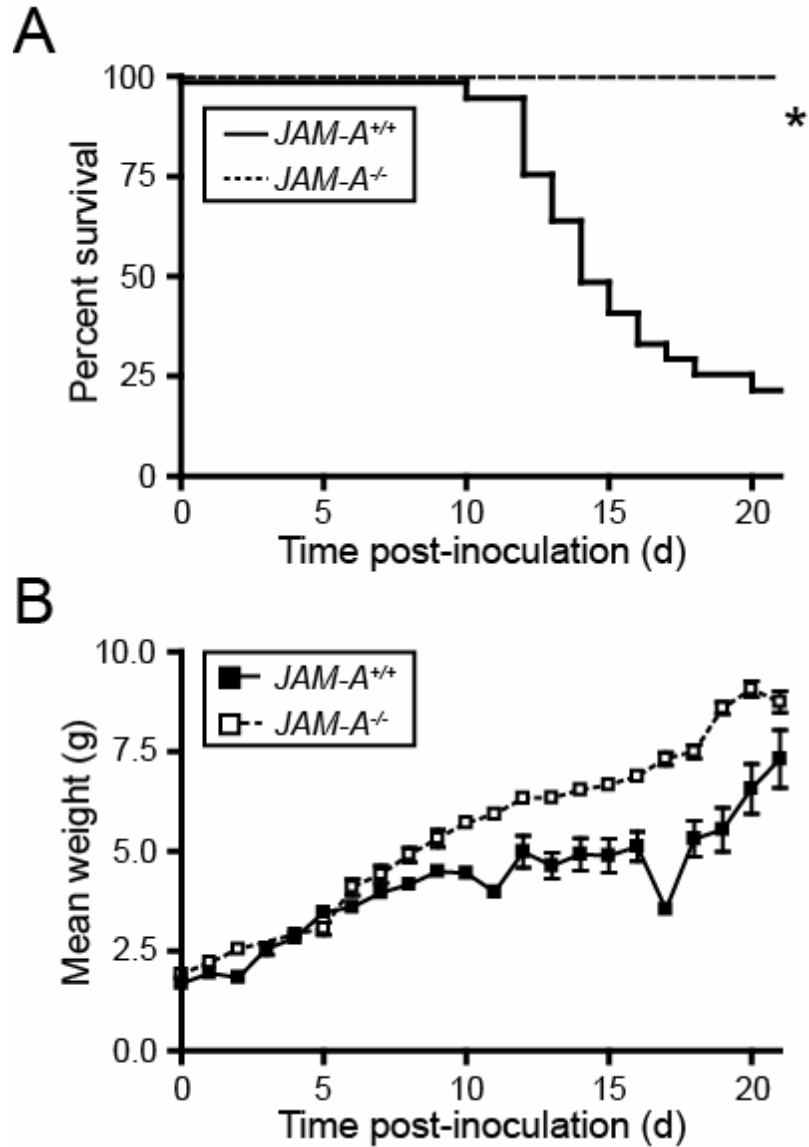


FIGURE II-3. T3SA- virulence is abolished following peroral inoculation of *JAM-A*^{-/-} mice. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁹ PFU of reovirus T3SA-. Mice (n = 26 *JAM-A*^{+/+} and n = 16 *JAM-A*^{-/-}) were monitored for survival (A) and weight gain (B). *, *P* < 0.0001 as determined by log rank test.

infection, including the brain, heart, liver, lung, and spleen, were substantially greater in wild-type animals than in *JAM-A*^{-/-} mice (Figure II-4). Thus, following peroral inoculation, JAM-A is dispensable for T3SA- replication in the intestine but is required

for production of maximal reovirus titer at sites of secondary infection, including the CNS.

JAM-A is required for systemic reovirus replication following peroral inoculation of T1L - T1 and T3 reovirus strains exhibit differential tropism and disseminate via different routes (85, 136, 148, 149). Since T1L does not produce uniformly lethal disease (148), survival studies using this strain were not possible. Nonetheless, to determine whether JAM-A is required for systemic replication of a T1 reovirus strain, I inoculated wild-type and *JAM-A*^{-/-} mice perorally with 10⁶ PFU T1L and quantified viral titer in multiple organs at various intervals post-inoculation. As with T3SA-, I found that the titer of T1L in the intestine of *JAM-A*^{-/-} mice was similar to that in wild-type animals (Figure II-5). Titers of T1L at all other sites were substantially greater in wild-type animals than in *JAM-A*^{-/-} mice. T1L titers in the liver, lung, and spleen of *JAM-A*^{-/-} animals were very low at 4 and 8 days post-inoculation, and, unlike T3SA-, T1L titer was absent from the heart and brain at all timepoints tested (Figure II-5). Taken together, these results indicate that JAM-A is required for systemic infection following peroral inoculation of reovirus T1L.

JAM-A is required for systemic reovirus replication following peroral inoculation of T3SA+ - Reovirus strains T3SA- and T3SA+ differ by a single amino acid polymorphism in $\sigma 1$ that confers the capacity to bind sialic acid (3). To determine whether JAM-A is required for systemic replication of a T3 reovirus strain that utilizes sialic acid as a receptor, I inoculated wild-type and *JAM-A*^{-/-} mice perorally with 10⁴ PFU T3SA+ and

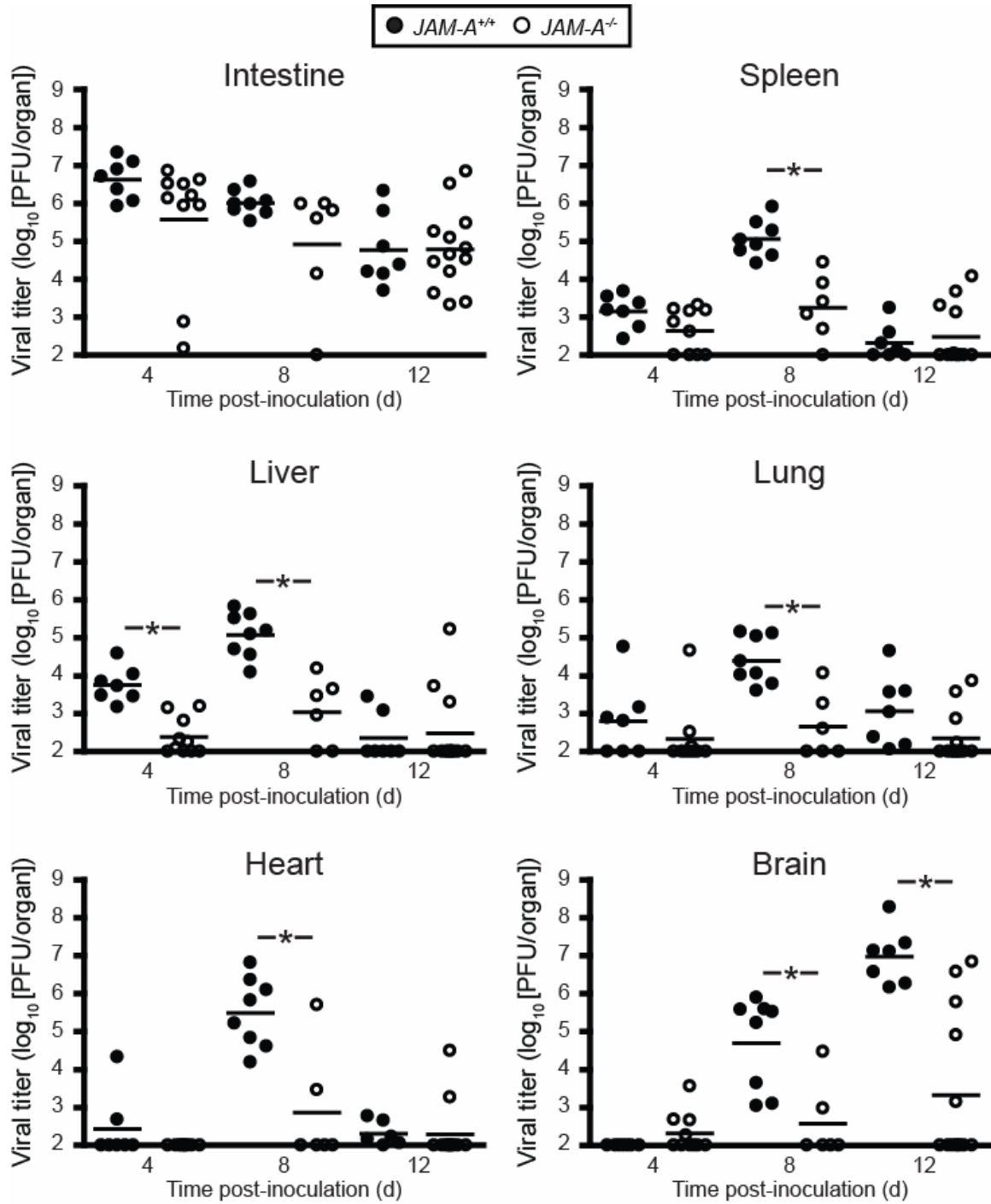


FIGURE II-4. Systemic T3SA- replication is attenuated following peroral inoculation of *JAM-A*^{-/-} mice. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁴ PFU T3SA-. At days 4, 8, and 12 after inoculation, mice were euthanized, organs were resected, and viral titers were determined by plaque assay. Each data point represents one animal. Horizontal bars indicate the arithmetic mean of log-transformed data. *, *P* < 0.05 by Student's *t* test.

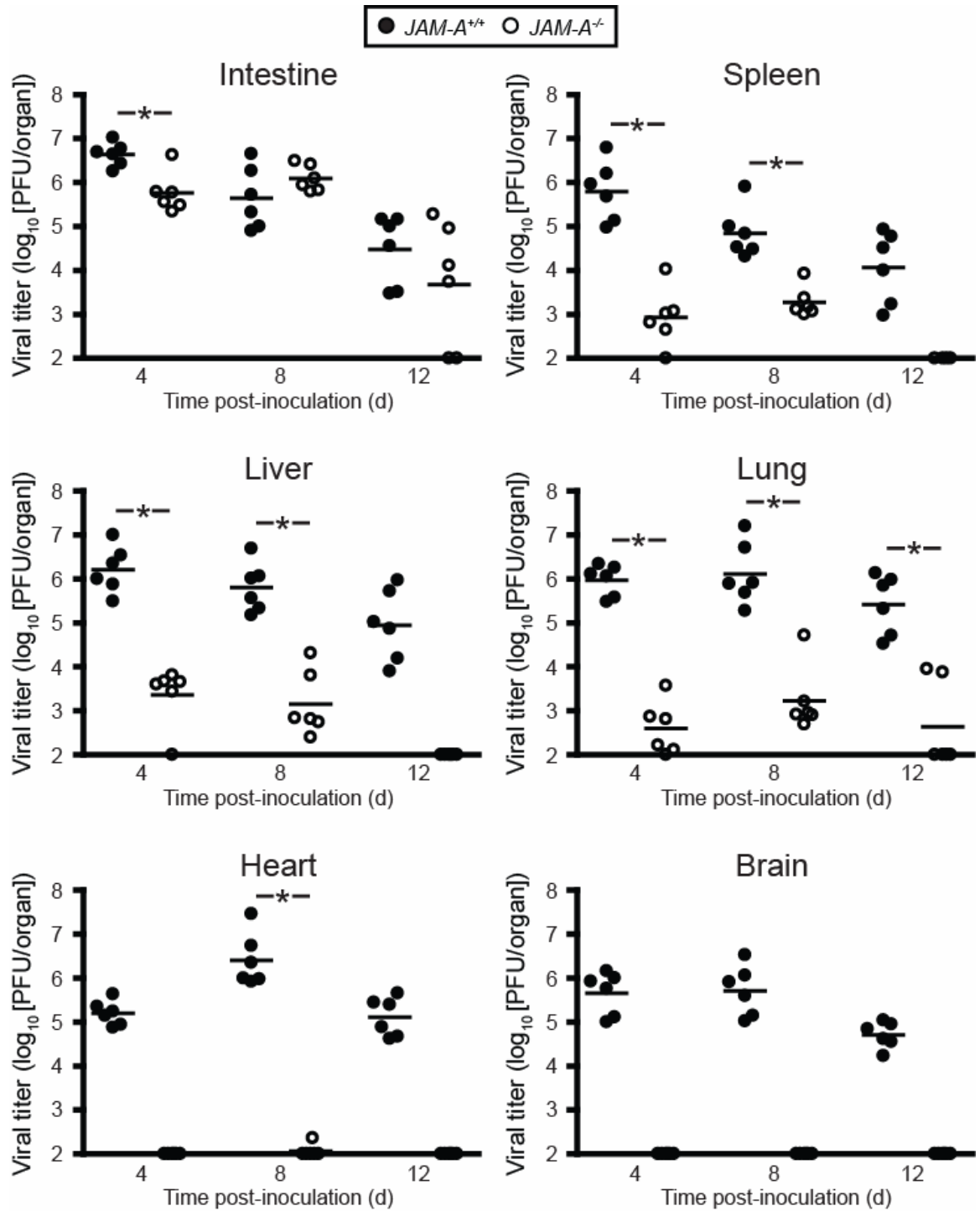


FIGURE II-5. Systemic T1L replication is attenuated following peroral inoculation of *JAM-A*^{-/-} mice. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁶ PFU T1L. At days 4, 8, and 12 after inoculation, mice were euthanized, organs were resected, and viral titers were determined by plaque assay. Each data point represents one animal. Horizontal bars indicate the arithmetic mean of log-transformed data. *, *P* < 0.05 by Student's *t* test. When all values are less than the limit of detection, a Student's *t* test *P* value cannot be calculated.

quantified viral titer at various intervals post-inoculation. As with T3SA-, I found that titers of T3SA+ in the intestine of *JAM-A*^{-/-} mice were similar to those in wild-type animals (Figure II-6). Titers of T3SA+ at all other sites at day 8 were substantially greater in wild-type animals than in *JAM-A*^{-/-} mice. T3SA+ titers in the liver of *JAM-A*^{-/-} mice are greater than T3SA- titers in the liver of *JAM-A*^{-/-} mice (Figures II-4 and II-6), suggesting that sialic acid serves as a reovirus receptor in the mouse liver. Unlike results with T3SA-, in which several *JAM-A*^{-/-} animals harbored replication in the CNS, only one *JAM-A*^{-/-} animal at each timepoint displayed T3SA+ titers in the brain. These results demonstrate that JAM-A is required for systemic infection following peroral inoculation of T3SA+. Thus, JAM-A is a requirement for systemic infection of both T1 and T3 reoviruses.

Discussion

In this chapter, I present data indicating that genetic deletion of JAM-A in fibroblast cells results in a 100- to 1,000-fold decrease in reovirus infectivity and replication. I demonstrate further that the residual T3 reovirus infection of JAM-A-null fibroblasts is at least in part due to reovirus interactions with cell-surface sialic acid. However, these data also indicate that some reovirus infection of *JAM-A*^{-/-} MEFs is mediated neither by host cell sialic acid nor by the viral 9BG5-binding region of the T3 σ 1 head. Although JAM-A is the only proteinaceous receptor known to bind to the reovirus σ 1 head domain, it is plausible that reovirus infection of some types of cells occurs via interactions with receptors that bind regions of reovirus σ 1 outside the 9BG5-

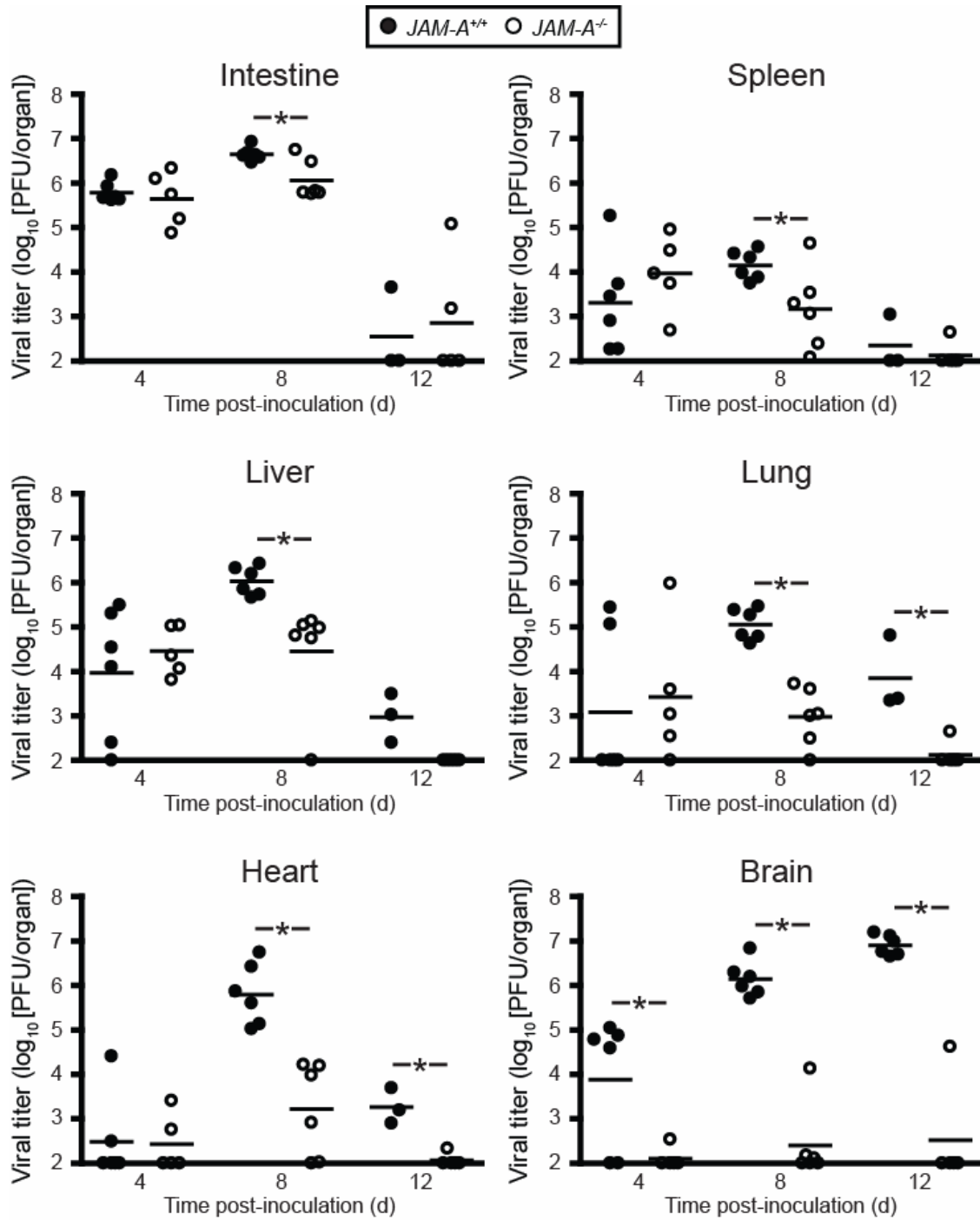


FIGURE II-6. Systemic T3SA+ replication is attenuated following peroral inoculation of *JAM-A*^{-/-} mice. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁴ PFU T3SA+. At days 4, 8, and 12 after inoculation, mice were euthanized, organs were resected, and viral titers were determined by plaque assay. Each data point represents one animal. Horizontal bars indicate the arithmetic mean of log-transformed data. *, *P* < 0.05 by Student's *t* test. When all values are less than the limit of detection, a Student's *t* test *P* value cannot be calculated.

binding area or other exposed viral proteins. It is also possible that reovirus infection of cells occurs via a receptor-independent uptake mechanism.

By virtue of their capacity to infect the intestine and spread systemically to the CNS, reoviruses offer an ideal experimental system to define how neurotropic viruses interact with their hosts at different steps during the disease process. Following peroral inoculation, reovirus T3SA- interactions with JAM-A are required for replication in target organs and production of lethal encephalitis but surprisingly dispensable for intestinal replication. Similarly, JAM-A is also required for systemic replication of both T1L and T3SA+ reovirus strains. These results indicate that JAM-A serves to promote systemic infection of reovirus in a serotype-independent manner.

A striking finding of these studies is that genetic deletion of JAM-A does not influence the capacity of reovirus to replicate in the intestine following peroral inoculation. This is true for the T3SA-, T3SA+, and T1L strains of reovirus. Previous work has demonstrated that the capacity to bind sialic acid does not influence reovirus replication in the intestine following peroral inoculation (5). Therefore, neither JAM-A nor sialic acid is likely the cellular ligand that mediates reovirus replication in the mouse intestine. Since both T1 and T3 reovirus strains can replicate in the intestine of *JAM-A*^{-/-} mice, it is likely that reovirus infection at that site is mediated by a serotype-independent receptor or a receptor-independent uptake mechanism.

Whereas T1 and T3 reoviruses replicate to high titer in the intestine of both wild-type and *JAM-A*^{-/-} mice, replication at extra-intestinal sites is severely curtailed in JAM-A-null mice in comparison to wild-type mice. I envision two possible explanations for this finding. First, JAM-A might be required for replication in target organs. In this case,

JAM-A would be the primary reovirus receptor at sites outside the intestine, whereas an unknown serotype-independent receptor or a receptor-independent uptake mechanism would mediate reovirus replication in the intestine. Second, JAM-A might be required for dissemination from the intestine to target organs. Since reovirus can disseminate neurally, hematogenously, or via both pathways, in this scenario JAM-A would be required for reovirus dissemination via one or both of these routes. By circumventing dissemination pathways altogether, intracranial inoculation experiments using wild-type *JAM-A*^{-/-} mice are required to discriminate between these possibilities.

CHAPTER III

JAM-A IS NOT REQUIRED FOR REOVIRUS INFECTION OF NEURONS OR TROPISM WITHIN THE CNS

Introduction

In Chapter II, I describe work demonstrating that JAM-A is not required for reovirus replication in the intestine but is required for systemic replication of reovirus. I thought it possible that reovirus might be incapable of either dissemination to sites of secondary infection in *JAM-A*^{-/-} mice or establishing infection once reaching those sites. To distinguish between these possibilities, I inoculated wild-type and isogenic JAM-A-null mice intracranially. I found that JAM-A is not required for reovirus virulence or replication in the CNS following intracranial inoculation. I also found that JAM-A is not required for T3 reovirus tropism for neurons in the cortex, hippocampus, thalamus, and cerebellar Purkinje cells. Further, JAM-A is not required for reovirus infection of primary cortical neurons in vitro. Together with findings from Chapter II, these results reveal a role for JAM-A in systemic dissemination of reovirus and provide evidence for the existence of a neural-specific T3 reovirus receptor.

Some of the research described in this chapter was performed in collaboration with other investigators. Dr. Ty Abel (Department of Pathology at the Vanderbilt University School of Medicine) guided the histologic analyses of brain sections and captured representative images. Ana Perdigoto and Dr. Bruce Carter (Department of Biochemistry and Center for Molecular Neuroscience at the Vanderbilt University School of Medicine) aided in the extraction and culture of primary cortical neurons from wild-

type and *JAM-A*^{-/-} mouse embryos. Dr. Jennifer Konopka (Department of Pediatrics at the Vanderbilt University School of Medicine) and Johnna Allen (Department of Microbiology & Immunology at the Vanderbilt University School of Medicine) assisted in the extraction and culture of mouse cortical neuron cultures. Dr. Konopka and I contributed equally to the cortical neuron studies presented here. In addition, Dr. Konopka performed the blinded analysis of histologic sections presented in Table III-2.

Results

JAM-A is not required for lethal reovirus infection following intracranial inoculation - To determine whether JAM-A is required for reovirus virulence once virus accesses the brain, I inoculated wild-type and *JAM-A*^{-/-} mice intracranially with escalating doses of reovirus T3SA- and assessed survival as a function of dose. Both wild-type and *JAM-A*^{-/-} mice succumbed to lethal encephalitis, yielding similar LD₅₀ values (Table III-1). At a dose of 100 PFU T3SA- delivered intracranially, wild-type and *JAM-A*^{-/-} mice alike succumbed to lethal reovirus-induced disease (Figure III-1 A). Alterations in weight gain following infection of the two genotypes were equivalent (Figure III-1 B). These results indicate that reovirus is fully virulent following direct inoculation into the brain of *JAM-A*^{-/-} mice and suggest a role for JAM-A in systemic dissemination of reovirus from the intestine.

JAM-A is not required for T1 and T3 reovirus replication in the brain following intracranial inoculation - Following peroral inoculation, JAM-A is required for systemic replication of both T1 and T3 reovirus strains (Figures II-4, II-5, and II-6). In particular,

TABLE III-1. JAM-A is dispensable for T3SA- virulence following intracranial inoculation^a

Dose (PFU/mouse)	<i>JAM-A</i> ^{+/+}		<i>JAM-A</i> ^{-/-}	
	Survive	Succumb	Survive	Succumb
1	17	0	16	0
10	10	1	11	3
10 ²	9	31	5	27
10 ³	1	21	0	14
10 ⁴	0	16	0	13

^aNewborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated intracranially with the indicated doses of T3SA-. Each dose was used to inoculate 2-3 litters of mice. Animals were monitored for survival for 21 days after inoculation, at which point survivors appeared healthy. LD₅₀ values were calculated according to the method of Reed and Muench (104). The LD₅₀ value of T3SA- in wild-type mice is 52 PFU. The LD₅₀ value of T3SA- in *JAM-A*^{-/-} mice is 41 PFU.

replication of T1 and T3 reovirus strains in the brain of *JAM-A*^{-/-} mice following peroral inoculation is diminished in comparison to that in wild-type animals. To determine whether JAM-A is required for reovirus replication in the brain, I inoculated wild-type and *JAM-A*^{-/-} mice intracranially with 100 PFU T3SA- or 10³ PFU T1L and quantified viral titer in brain homogenates at various intervals post-inoculation. I found that T3SA- and T1L reached equivalent or higher titers in the brain of *JAM-A*^{-/-} mice in comparison to those in wild-type mice following intracranial inoculation (Figure III-2). These results indicate that murine CNS tissue is capable of supporting T1L and T3SA- reovirus infection regardless of JAM-A expression.

To determine whether sialic acid-binding T3 reovirus strains can replicate in *JAM-A*^{-/-} CNS tissue, I inoculated wild-type and *JAM-A*^{-/-} mice perorally with 100 PFU of either T3D or T3SA+ and quantified viral titer in brain homogenates. I found that T3SA+ reached equivalent or higher titer in the brain of *JAM-A*^{-/-} mice in comparison to

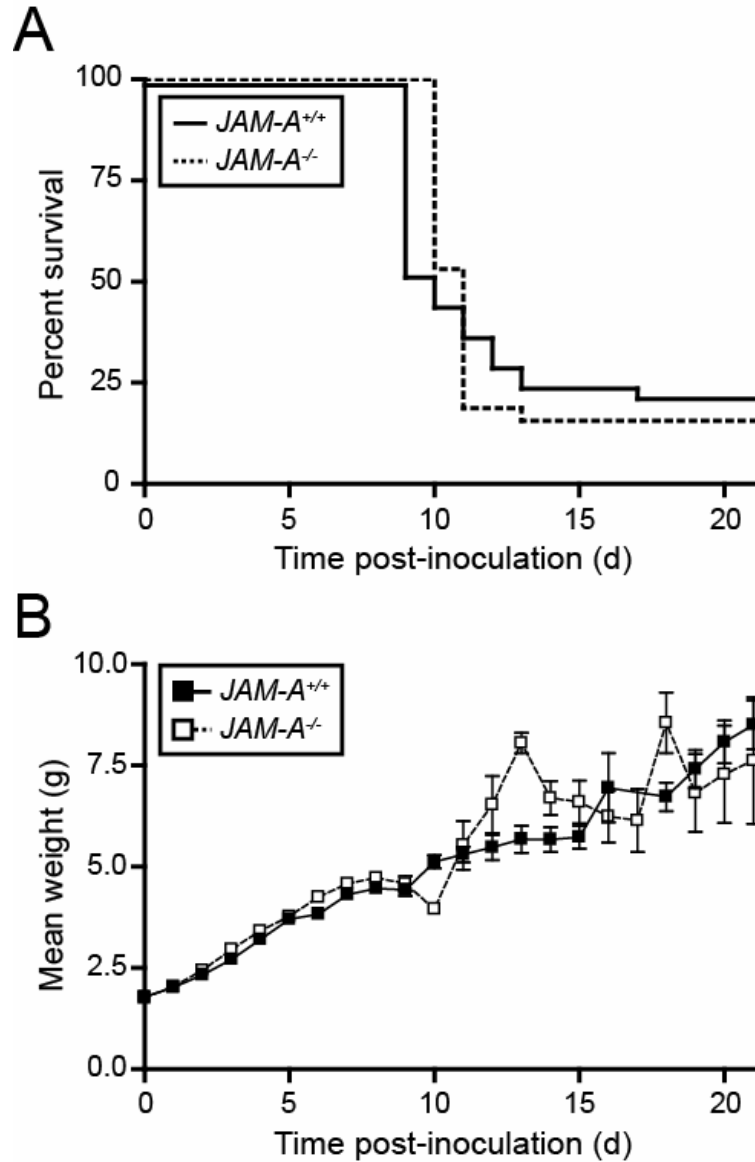


FIGURE III-1. T3SA- is fully virulent following intracranial inoculation of *JAM-A*^{-/-} mice. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated intracranially with 100 PFU of reovirus T3SA-. Mice (n = 15 *JAM-A*^{+/+} and n = 13 *JAM-A*^{-/-}) were monitored for survival (A) and weight gain (B). Survival *P* value > 0.05 as determined by log rank test.

that in wild-type mice following intracranial inoculation (Figure III-2). However, T3D replicated somewhat less efficiently in *JAM-A*^{-/-} mouse brain compared to wild-type mouse brain (Figure III-2). These results indicate that sialic acid-binding T3 reovirus

strains can replicate to high titers in the brain of *JAM-A*^{-/-} animals. Taken together with results from Chapter II, these data indicate that JAM-A is required for systemic dissemination of T1 and T3 reovirus strains from the intestine.

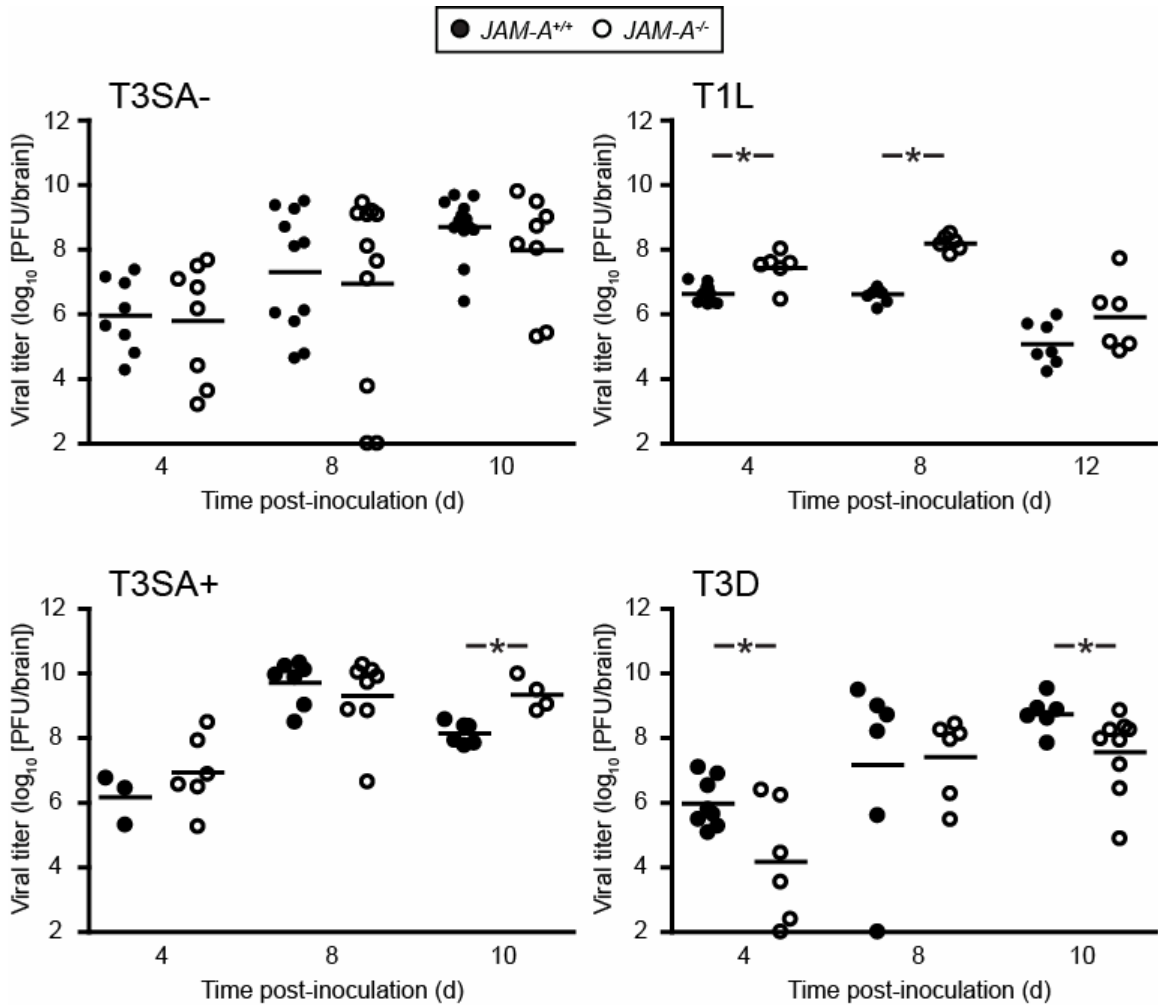


FIGURE III-2. Reovirus replicates to high titer in the brains of *JAM-A*^{-/-} mice. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated intracranially with 100 PFU T3SA-, 10³ PFU T1L, 100 PFU T3SA+, or 100 PFU T3D. At days 4, 8, 10, and 12 after inoculation, mice were euthanized, brains were resected, and viral titers were determined by plaque assay. Each data point represents one animal. Horizontal bars indicate the arithmetic mean of log-transformed data. *, *P* < 0.05 by Student's *t* test.

T3 reovirus regional tropism in the brain is not altered in JAM-A^{-/-} mice - The $\sigma 1$ -encoding S1 gene of reovirus is the main determinant of viral tropism within the CNS (81, 86, 148, 150). To test whether $\sigma 1$ -JAM-A interactions are required for the distinct regional tropism of T3 reovirus, I compared histologic sections of brain from wild-type and *JAM-A^{-/-}* mice inoculated intracranially with 10^4 PFU T3SA-. Brains of infected mice were resected and bisected sagittally. The left hemisphere was prepared for viral titer determination by plaque assay, and the right hemisphere was processed for histopathology. Brain sections chosen for histologic analysis were matched for hippocampal depth and viral titer. Hematoxylin and eosin (H&E)-stained sections showed a consistent pattern of injury in both wild-type and *JAM-A^{-/-}* mice. In both mouse strains, neurons of the hippocampal pyramidal layers were affected, showing abundant eosinophilic, cytoplasmic inclusions and individual cell necrosis and apoptosis. Similar changes were observed in cerebellar Purkinje layer neurons, with relative sparing of granule cells. Other regions consistently affected in both strains of mice included the dorsal thalamic nuclei, hypothalamus, and middle layers of the cerebral cortex (Figure III-3). Immunohistochemistry for reovirus antigen confirmed the presence of virus in hippocampal and cerebellar neurons and cells with neuronal morphology in other affected regions (Figure III-3). Ependymal cells were spared in both genotypes of mice. The reovirus antigen signal appeared more intense in the cortex, especially the outer layers, and the hippocampus of *JAM-A^{-/-}* brain when compared to wild-type brain. To quantify this observation, we performed a blinded analysis of brain sections matched for hippocampal depth and viral titer and found that a greater percentage of cells in both the middle and outer layers of cells of the cortex displayed reovirus antigen in *JAM-A^{-/-}* mice compared to wild-type (Table III-2).

However, reovirus targeted the same cell types and brain regions in both mouse strains. These data indicate that JAM-A is not the primary cellular factor mediating the unique targeting of T3 reovirus to specific regions within the brain.

Reovirus infection of mouse cortical cultures does not depend on JAM-A - To directly test whether JAM-A is required for reovirus infection of neurons, I infected primary cortical neuron cultures prepared from embryonic wild-type and *JAM-A*^{-/-} mice with T1 and T3 reovirus strains. The cortical neurons displayed classic neuronal morphology and stained for the neuron-specific beta III tubulin marker, TUJ1. The pattern of T3 reovirus antigen staining was identical in wild-type and *JAM-A*^{-/-} cortical neurons, which displayed similar morphology and characteristics of reovirus infection (Figure III-4 A). In both types of neurons, reovirus-positive inclusions were observed dotting the length of infected axons, a staining pattern consistent with focal sites of reovirus replication (9). T3D replication in neurons was equivalent in wild-type and *JAM-A*^{-/-} cultures (Figure III-4 B). T1L replicated less than T3D in mouse cortical neurons (Figure III-4 B), consistent with previous observations (35). Replication of T3SA⁻ and T3SA⁺ was diminished in *JAM-A*^{-/-} neuron cultures compared to wild-type at 24 hours but was equivalent to wild-type by 48 hours post-infection. Thus, JAM-A is dispensable for neural infection of T3 reovirus strains.

T3 reovirus infection of primary cortical neurons is reduced following treatment with neuraminidase and $\sigma 1$ antibody - To determine whether sialic acid binding mediates viral replication in primary cortical neurons, I assessed reovirus infection at 20 h following

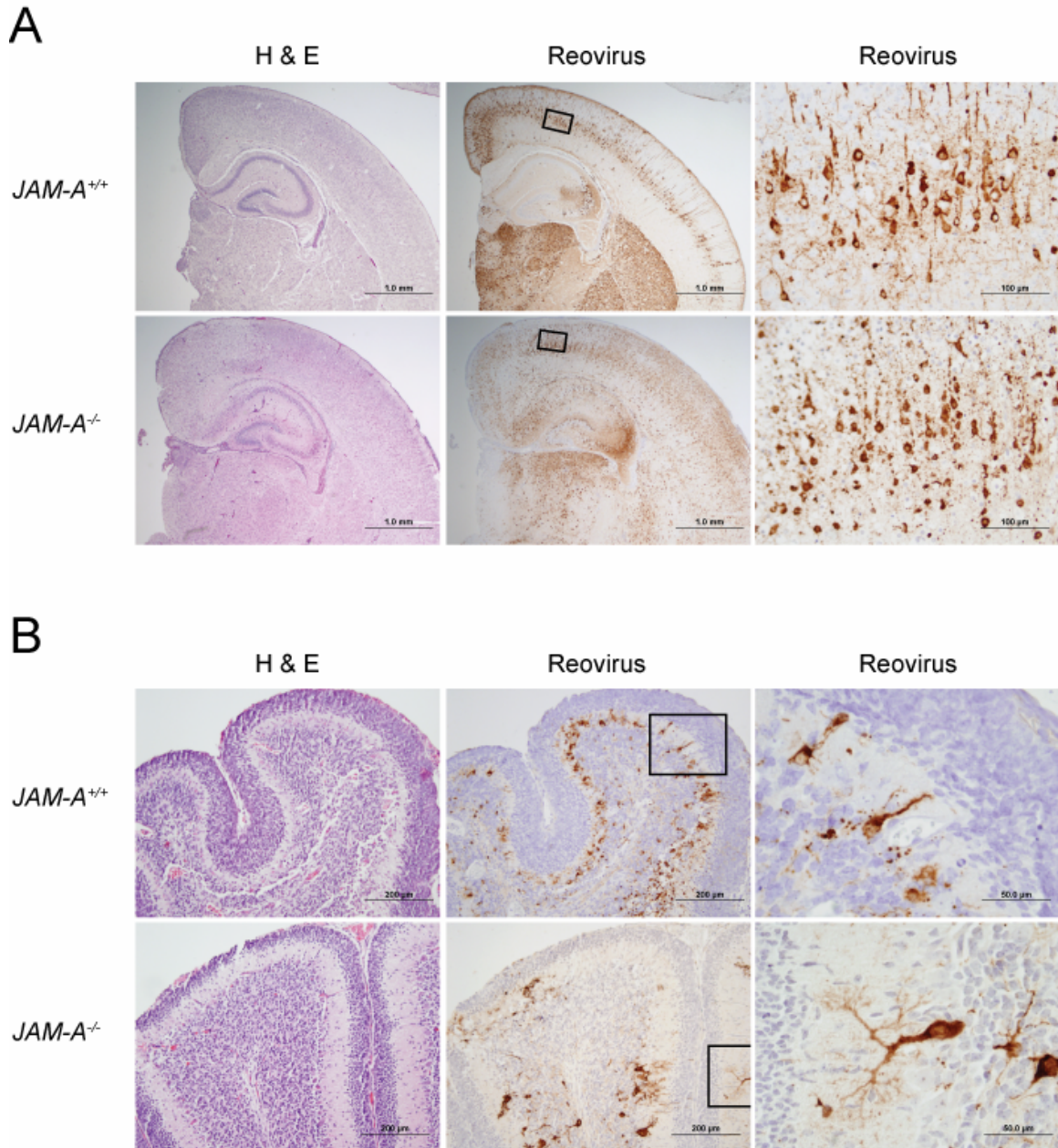


FIGURE III-3. *JAM-A* is dispensable for T3SA- tropism within the brain. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated intracranially with 10⁴ PFU T3SA-. Eight days after inoculation, brains of infected mice were resected and bisected sagittally. The left hemisphere was prepared for viral titer determination by plaque assay, and the right hemisphere was processed for histopathology. Consecutive coronal sections were stained with H&E or polyclonal reovirus antiserum. Representative sections of brain hemisphere (A), matched for hippocampal depth, and cerebellum (B) are shown. Boxes indicate areas of enlargement in the panels on the right and show cortical neurons (A) and cerebellar Purkinje neurons (B). *JAM-A*^{+/+} brain sections are from brains with left hemisphere viral titers of 4.1 x 10⁹ PFU (A) and 3.0 x 10⁹ (B). *JAM-A*^{-/-} brain sections are from brains with left hemisphere viral titers of 3.4 x 10⁹ PFU (A) and 1.6 x 10⁹ PFU (B).

TABLE III-2. JAM-A deletion alters the reovirus antigen staining intensity in the cortex following intracranial inoculation^a

Genotype	Viral Titer	Cortical Layer		Hippocampal Region		Thalamus	Cerebellar Purkinje Layer
		Middle	Outer	CA 1	CA 2-4		
<i>JAM-A</i> ^{+/+}	9.53	2	1	1	2	4	5
	9.61	3	1	1	2	4	2
	9.48	1	0	1	2	3	4
	9.49						4
	9.75						4
Group mean	9.57	2	0.67	1	2	3.67	3.8
<i>JAM-A</i> ^{-/-}	9.02	5	4	2	4	4	1
	9.48	5	3	1	2	2	3
	9.60	5	5	3	4	5	5
	8.89	3	3	1	3	1	1
	9.53	4	3	4	4	2	1
	9.19						2
Group mean	9.28	4.4	3.6	2.2	3.4	2.8	2.17
	<i>P</i> > 0.05	<i>P</i> < 0.05	<i>P</i> < 0.05	#	#	<i>P</i> > 0.05	<i>P</i> > 0.05

^aNewborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated intracranially with 10⁴ PFU T3SA-. Eight days after inoculation, brains of infected mice were resected and bisected sagittally. The left hemisphere was prepared for viral titer determination by plaque assay, and the right hemisphere was processed for histopathology. Brain sections chosen for histologic analysis were matched for hippocampal depth. The researcher was blinded to the genotype of the brain section and scored each region according to the following scale: 0 indicates no reovirus antigen staining, 1 indicates 1-20% of cells in the indicated region display reovirus antigen staining, 2 indicates 21-40% of cells in the indicated region display reovirus antigen staining, 3 indicates 41-60% of cells in the indicated region display reovirus antigen staining, 4 indicates 61-80% of cells in the indicated region display reovirus antigen staining, and 5 indicates 81-100% of cells in the indicated region display reovirus antigen staining. When sections matched for hippocampal depth were not available for an animal, that animal was not included in the analysis. *P* values compare the *JAM-A*^{+/+} group to the *JAM-A*^{-/-} group and were calculated by Student's *t* test. # indicates that a Student's *t* test *P* value cannot be calculated because all values are identical for one group.

either mock treatment or treatment with *A. ureafaciens* neuraminidase to remove cell-surface sialic acid. T3 reovirus strains infected the primary neurons, whereas T1L infection was minimal, consistent with previous reports (35). T3D and T3SA+ infection of both wild-type and *JAM-A*^{-/-} neurons was reduced following treatment of cells with neuraminidase (Figure III-5). Consistent with previous findings using HeLa cells (3) and MEFs (Figure II-2), neuraminidase treatment did not decrease infection of primary neurons with T3SA- (Figure III-5). T3SA+ infection of neural cultures in the untreated condition was approximately three-fold higher than that of T3SA- (Figure III-5). These data indicate that T3D and T3SA+ reovirus replication in primary neurons is in part mediated by sialic acid.

The capacity of reovirus to replicate in primary neurons is mediated by the S1 gene, but the only known reovirus receptor that binds the $\sigma 1$ head, JAM-A, is not responsible for mediating reovirus infection of neurons (Figure III-4). It is possible that other cellular receptors also bind this $\sigma 1$ domain. To determine whether T3 reovirus replication in primary neurons depends on interactions of the $\sigma 1$ head with the cell surface, I assessed virus replication following incubation of virus and cells with the T3 $\sigma 1$ head-specific MAb 9BG5 (17) and the T1 $\sigma 1$ head-specific MAb 5C6 (142). Treatment with MAb 9BG5 substantially reduced infectivity of T3D and T3SA- reovirus strains in primary neurons (Figure III-5). T1L did not infect primary neurons regardless of treatment (Figure III-5). These data suggest that T3 reovirus infection of primary cortical neurons is mediated by a $\sigma 1$ head-specific receptor other than JAM-A.

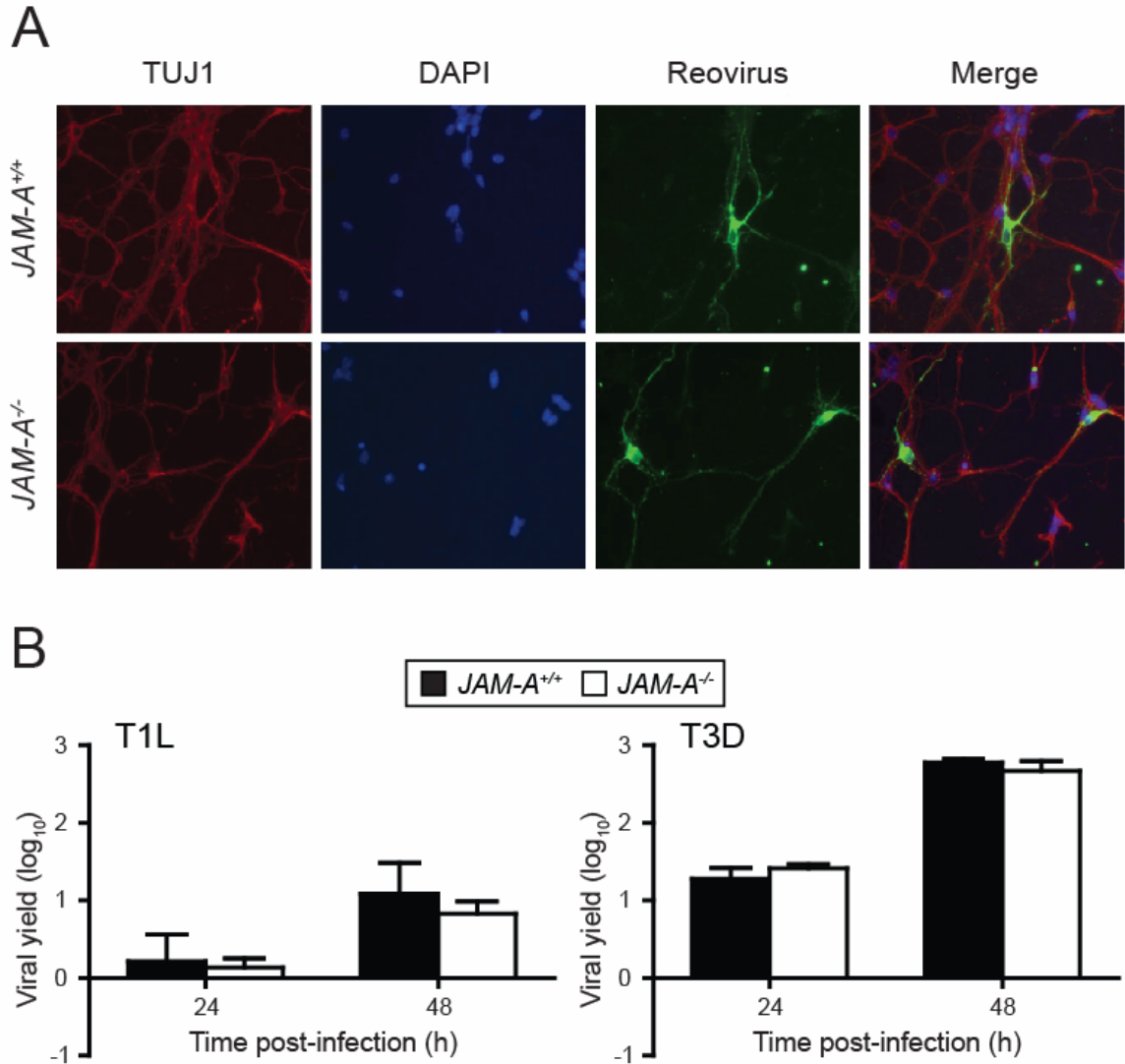


FIGURE III-4. JAM-A is not required for reovirus replication in primary cortical neurons. Cortices were isolated from day E15 *JAM-A*^{+/+} and *JAM-A*^{-/-} mice and cultured in vitro for 5 to 7 days. (A) Cells were infected with reovirus T3D at an MOI of 10³ PFU/cell. At 20 h post-infection, cells were fixed with methanol and stained with TUJ1 neural-specific marker to detect neurons (red), DAPI stain to detect nuclei (blue), and polyclonal reovirus antiserum to detect reovirus antigen (green). (B) Cells (2 × 10⁵) were adsorbed with T1L or T3D at an MOI of 10 PFU/cell. Titers of virus in cell lysates at the indicated intervals post-infection were determined by plaque assay. Results are expressed as viral yields for triplicate samples. Error bars indicate SD.

Discussion

Systemic virus infections are characterized by replication at the entry site followed by dissemination to and replication within target organs (137). In Chapter II, I present data indicating that reovirus interactions with JAM-A are required for replication in target organs and production of encephalitis but surprisingly dispensable for replication in the intestine. Here, I demonstrate that failure of reovirus to cause encephalitis in *JAM-A*^{-/-} mice following peroral inoculation is not due to an intrinsic block to reovirus replication in *JAM-A*^{-/-} neural tissues. Rather, JAM-A expression is required for efficient T1 and T3 reovirus dissemination in the host.

That JAM-A is not required for reovirus T3SA- replication in the brain suggests the existence of novel receptors for reovirus at this site. Analysis of histologic brain sections provides corroborating evidence indicating that T3SA- regional tropism within the CNS does not depend on JAM-A and is identical to the regional tropism of a sialic acid-binding T3 reovirus strain inoculated intracranially into wild-type mice (105). Furthermore, genetic deletion of JAM-A does not alter reovirus infection of primary cultures of neurons. These data provide strong evidence that reovirus interactions with neurons are mediated by a currently unknown serotype-specific receptor other than JAM-A or sialic acid.

Genetic deletion of *JAM-A* does not substantially alter the unique pattern of T3 reovirus tropism within the murine brain nor does it prevent the T1 and T3 reovirus strains tested here from replicating to high titer in the brains of mice. However, there exist subtle differences in reovirus tropism and replication in the two mouse strains. For example, T3 reovirus antigen staining is greater in the outer layers of the cortex of *JAM-A*^{-/-}

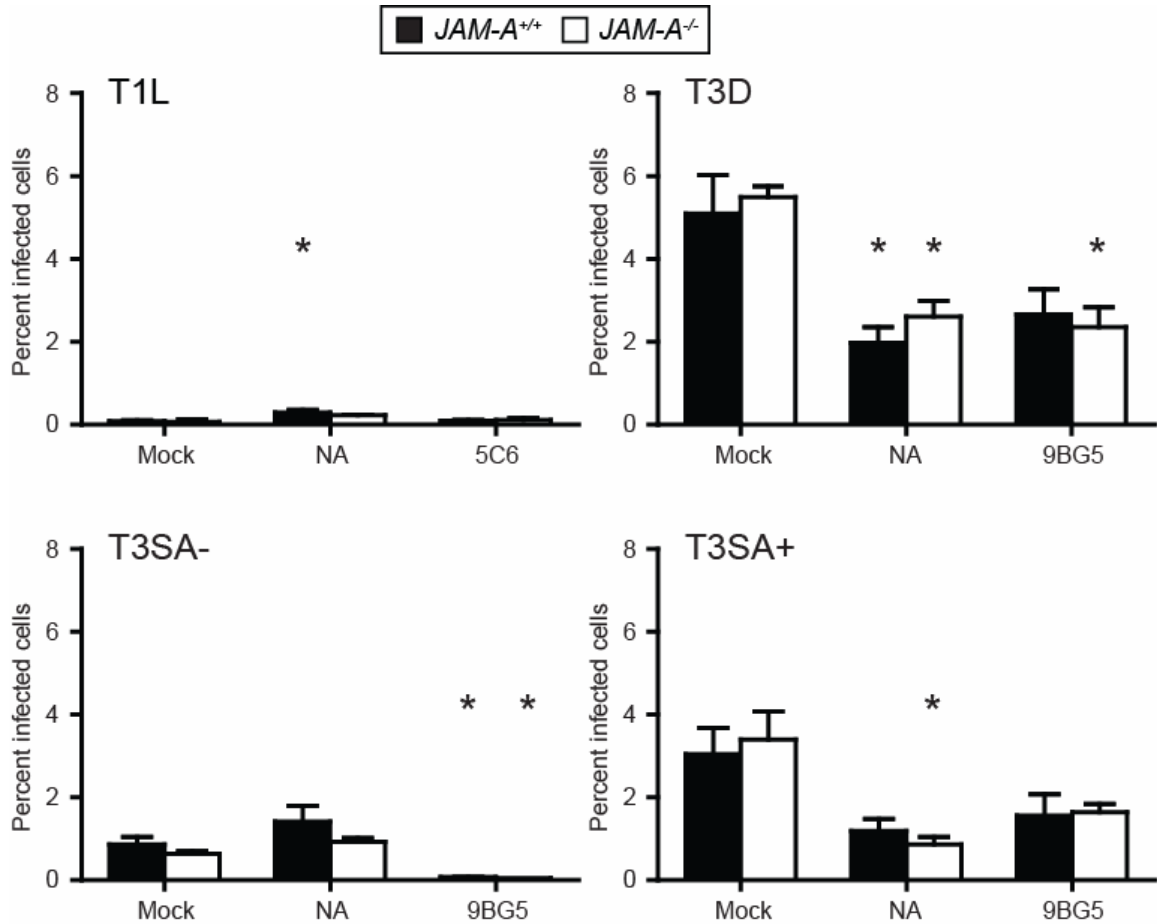


FIGURE III-5. Neuraminidase treatment and $\sigma 1$ antibody inhibit T3 infection of murine primary cortical cultures. Primary cortical cultures generated from *JAM-A*^{+/+} and *JAM-A*^{-/-} embryos were treated with incomplete media or 40 mU/mL neuraminidase for 1 h. Reovirus T1L was incubated with incomplete medium or 50 μ g/mL 5C6 for 1 h. T3D, T3SA-, and T3SA+ were incubated with incomplete medium or 50 μ g/mL 9BG5 for 1 h. Cells were adsorbed with reovirus at an MOI of 10^3 virus particles/cell and incubated for 20 h. Reovirus antigen and DAPI stain for nuclei were detected by indirect immunofluorescence. Total nuclei and infected cells were quantified in three fields of 200X view. Results are expressed as the mean percent infected cells/field for triplicate experiments. Error bars indicate SD. Within each condition, the *JAM-A*^{+/+} and *JAM-A*^{-/-} means are equivalent by Student's *t* test. *, $P < 0.05$ as determined by Student's *t* test compared to the genotype's mock condition.

mouse brains than in wild-type brains. Also, T1L brain replication is 10-50 fold greater following intracranial inoculation in *JAM-A*^{-/-} mouse brains than wild-type brains. It is possible that the absence of JAM-A results in alterations in expression of other reovirus

receptors in regions of *JAM-A*^{-/-} mouse brains. In support of this idea, overexpression of dimerization-defective JAM-A mutants in 293T cells results in diminished β 1 integrin expression (116).

In this chapter, I report that JAM-A is not required for T3SA- or T1L replication in the brain following intracranial inoculation, for the unique CNS tropism of T3SA-, or for T1 or T3 replication within primary neurons. These results, coupled with those presented in Chapter II, indicate that JAM-A is required for dissemination of reovirus from the intestine to target organs such as the brain.

CHAPTER IV

JAM-A IS REQUIRED FOR HEMATOGENOUS DISSEMINATION OF REOVIRUS

Introduction

Findings presented in Chapter II demonstrate that JAM-A is not required for reovirus growth in the intestine but is required for systemic replication of reovirus. In addition, work presented in Chapter III indicates that reovirus replication in the CNS is not dependent on JAM-A following intracranial inoculation, suggesting that JAM-A is required for efficient dissemination of reovirus from the intestine to target organs such as the brain. In newborn mice, reovirus can disseminate neurally or hematogenously, depending on the strain of reovirus and the route of inoculation. It was unknown whether JAM-A mediates neural, hematogenous, or both routes of dissemination.

In this study, I inoculated wild-type and isogenic JAM-A-null mice perorally and in the hindlimb and determined the extent to which JAM-A is required for reovirus replication in lymphatic tissue, establishment of viremia, and neural spread to the CNS. I found that JAM-A is not required for neural dissemination to the spinal cord, suggesting a role for JAM-A in mediating hematogenous dissemination. Indeed, JAM-A is required for infection of primary endothelial cells and establishment of high levels of reovirus viremia. These results provide strong evidence indicating that JAM-A is required for reovirus dissemination within the bloodstream.

Some of the research described in this chapter was performed in collaboration with other investigators. Dr. Ty Abel (Department of Pathology at the Vanderbilt

University School of Medicine) captured representative images of intestine. Dr. Ambra Pozzi (Departments of Cancer Biology and Medicine at the Vanderbilt University School of Medicine and Department of Medicine at the Nashville Veteran Affairs Hospital) guided the extraction and culture of primary murine lung endothelial cells.

Results

JAM-A promotes hematogenous but not neural spread of reovirus from the hindlimb to the spinal cord - Following inoculation into the hindlimb, section of the sciatic nerve ablates spread of strain T3D but not T1L to the inferior spinal cord (ISC), indicating that T3D spreads neurally to the spinal cord, whereas T1L spreads primarily hematogenously to that site (136). To determine the mechanism by which JAM-A promotes reovirus dissemination, we inoculated wild-type and *JAM-A*^{-/-} mice into the hindlimb with 10⁶ PFU of either T1L or T3D and quantified viral titer in the hindlimb, blood, and ISC. After inoculation with T3D, both wild-type and *JAM-A*^{-/-} mice harbored high viral titers in the ISC and low titers in the blood, consistent with an intact neural route of spread (Figure IV-1 A). The ratio of T3D titer in the ISC to that in the hindlimb in wild-type and *JAM-A*^{-/-} mice was equivalent at 2, 4, and 6 days post-inoculation, indicating that spread from hindlimb to ISC in both genotypes is intact at those times (Figure IV-1 B). On day 8, the ratio of T3D titer in the ISC to that in the hindlimb in *JAM-A*^{-/-} mice is lower than that in wild-type mice (Figure IV-1 B), perhaps reflecting accelerated clearance of T3D in the spinal cord of *JAM-A*^{-/-} mice in comparison to wild-type animals. After inoculation of T1L into wild-type mice, titers in blood and ISC increased virtually simultaneously over the experimental timecourse, consistent with a hematogenous route of spread (Figure IV-1 A).

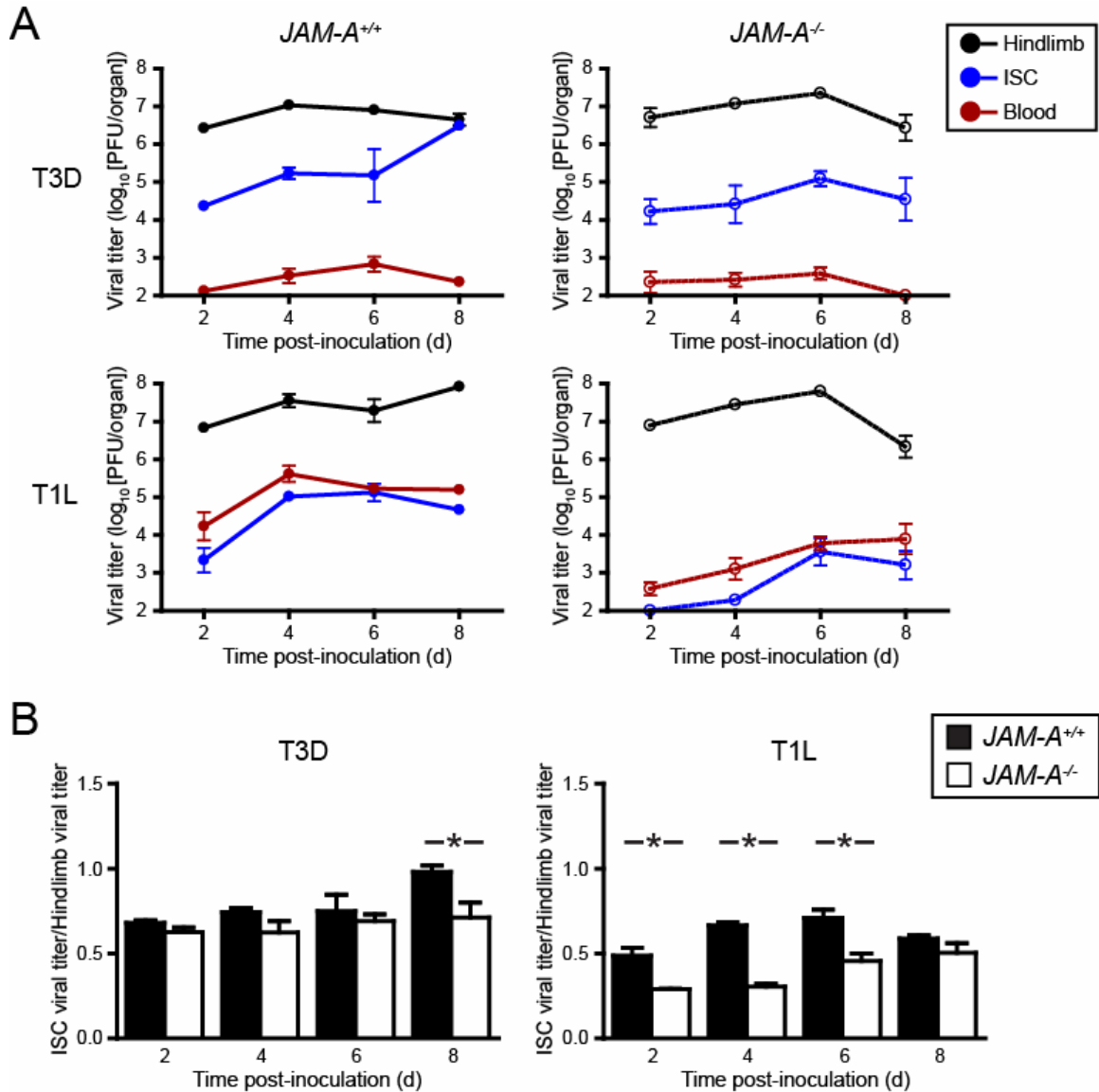


FIGURE IV-1. Following hindlimb inoculation, JAM-A is required for efficient hematogenous but not neural spread of reovirus. (A-B) Newborn *JAM-A^{+/+}* and *JAM-A^{-/-}* mice were inoculated into the left hindlimb with 10^6 PFU of either T3D or T1L. At days 2, 4, 6, and 8 after inoculation, mice were euthanized and left hindlimb, blood, and inferior spinal cord (ISC), including the thoracic and lumbosacral cord segments, were resected. Viral titers were determined by plaque assay. Results are expressed as mean viral titers (A) or as a ratio of ISC viral titer to hindlimb viral titer (B) for 6 animals for each time point. Error bars indicate SD. *, $P < 0.05$ by Student's *t* test.

In contrast, the titer of T1L in blood and ISC in *JAM-A*^{-/-} mice increased with markedly reduced kinetics in comparison to the titer rise in wild-type animals (Figure IV-1 A). The ratio of T1L titer in the ISC to that in the hindlimb in wild-type mice is greater than that in *JAM-A*^{-/-} mice at 2, 4, and 6 days post-inoculation, indicating that spread of T1L from hindlimb to ISC is blunted in *JAM-A*^{-/-} mice (Figure IV-1 B). These findings suggest that JAM-A is an important mediator of hematogenous but not neural dissemination of reovirus from the hindlimb.

Reovirus T1L targets intestinal epithelial cells and Peyer's patches regardless of JAM-A expression - To confirm that JAM-A is required for T1L reovirus replication in the intestine, we compared histologic sections of intestines from wild-type and *JAM-A*^{-/-} mice inoculated perorally with 10⁸ PFU of T1L. In both mouse strains, epithelial cells of small intestinal villi were infected at early times post-inoculation and showed abundant eosinophilic, cytoplasmic inclusions (Figure IV-2 A). Immunohistochemistry for reovirus antigen confirmed the presence of virus in villus epithelial cells (Figure IV-2 A). Mononuclear cells within Peyer's patches also displayed reovirus antigen (Figure IV-2 B). These results indicate that reovirus T1L replication in intestinal epithelial cells and intestinal lymphatic tissue is independent of JAM-A.

Reovirus dissemination to lymphatic tissues is diminished but not abolished in JAM-A^{-/-} *animals* - Following peroral inoculation and localization to Peyer's patches, reovirus T1L disseminates to mesenteric lymph nodes (MLNs) and spleen within 24 h (61). To determine whether JAM-A is required for lymphatic or bloodstream dissemination, we inoculated wild-type and *JAM-A*^{-/-} mice perorally with 10⁸ PFU of T1L and quantified viral

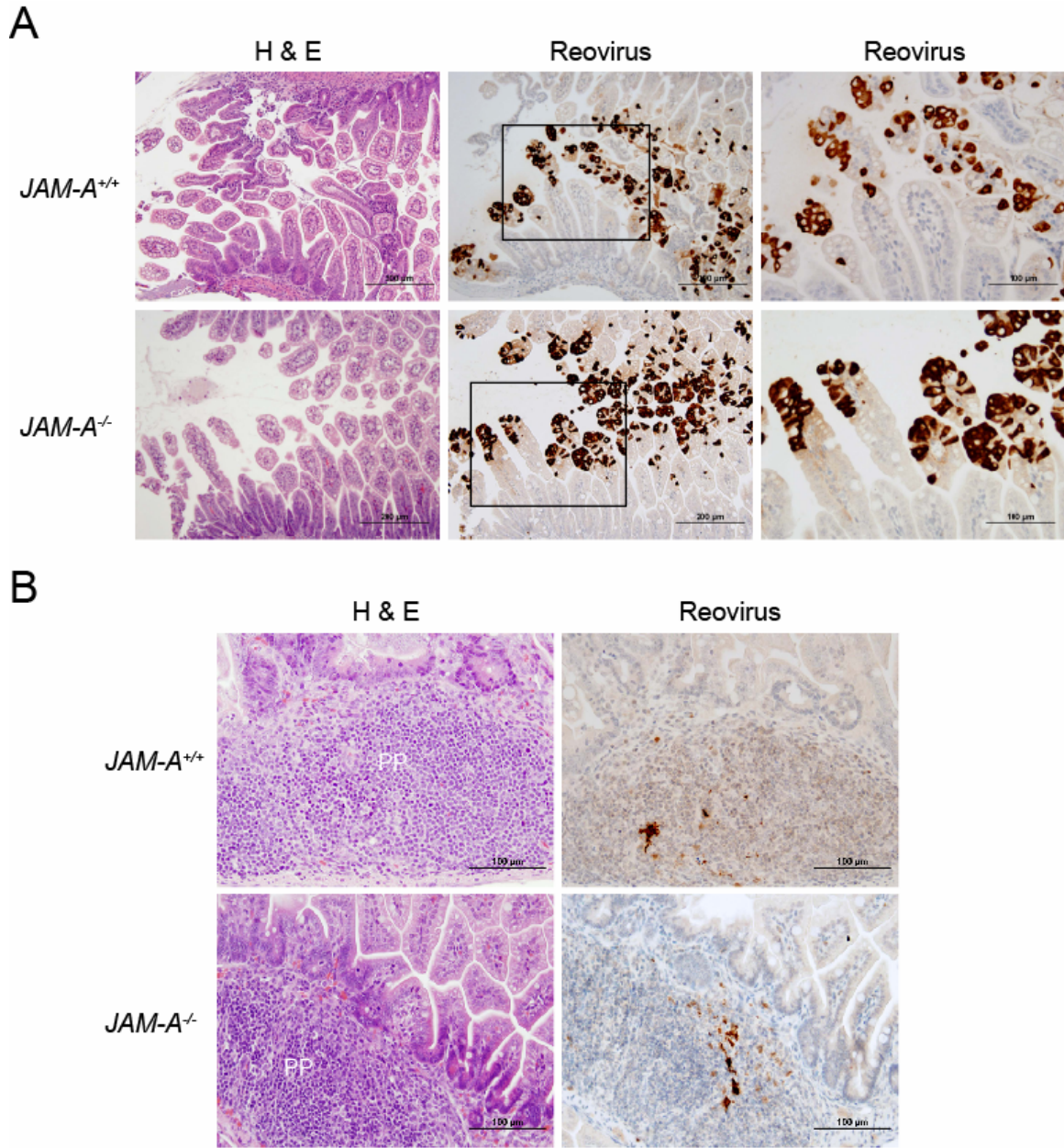


FIGURE IV-2. JAM-A is dispensable for tropism of reovirus T1L within the intestine. (A-B) Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁸ PFU of T1L. At days 2 and 4 after inoculation, small intestines were resected and processed for histopathology. Consecutive sections were stained with H&E or polyclonal reovirus antiserum. Representative sections of intestinal villi at day 2 (A) and Peyer's patches (PP) at day 4 (B) are shown. Boxes indicate areas of enlargement in the panels on the right and show villus epithelial cells.

titer in the MLN, spleen, and blood. In both wild-type and *JAM-A*^{-/-} mice, viral titer in the MLN and spleen were detectable at early times post-inoculation and increased over the course of infection, although T1L produced greater titers in wild-type versus *JAM-A*^{-/-} mice (Figure IV-3). The titer of T1L in the blood of wild-type mice was detectable by day 2 post-inoculation and increased thereafter (Figure IV-3). In sharp contrast, with the exception of one animal per timepoint, there was no detectable titer of T1L in the blood of *JAM-A*^{-/-} mice (Figure IV-3). Similar results were obtained in experiments using T3SA- (Figure IV-4). Therefore, JAM-A is not required for access to lymphatic routes of dissemination but is required for the establishment of high titer viremia following peroral inoculation of reovirus.

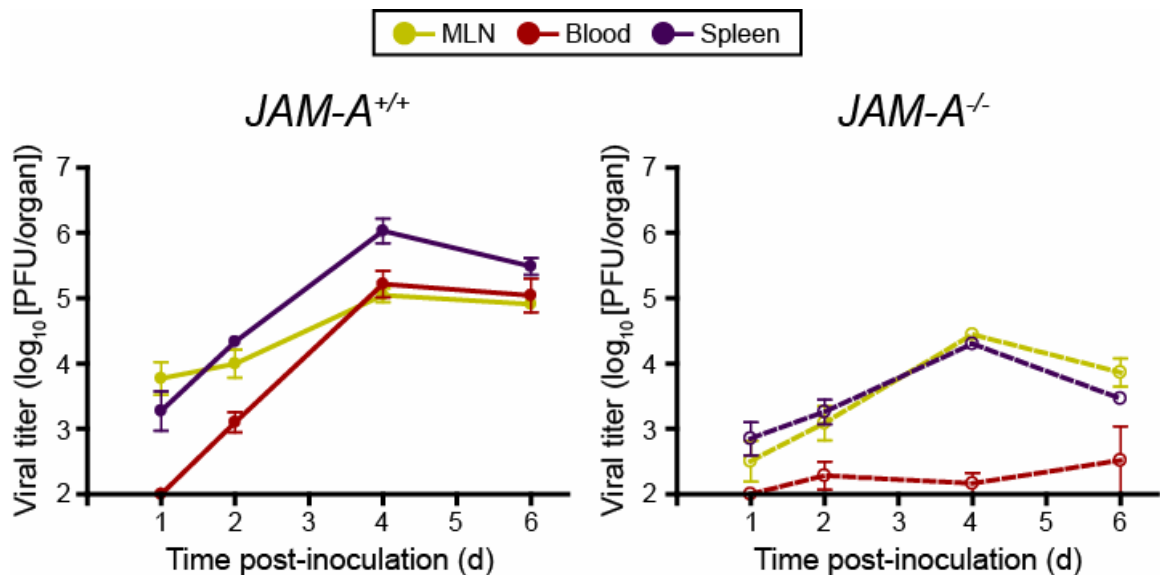


FIGURE IV-3. JAM-A is not required for reovirus dissemination to lymphatic tissue. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁸ PFU of T1L. At days 1, 2, 4, and 6 after inoculation, mice were euthanized, mesenteric lymph node (MLN), blood, and spleen were collected, and viral titers were determined by plaque assay. Results are expressed as mean viral titers for 3-8 animals for each time point. Error bars indicate SD.

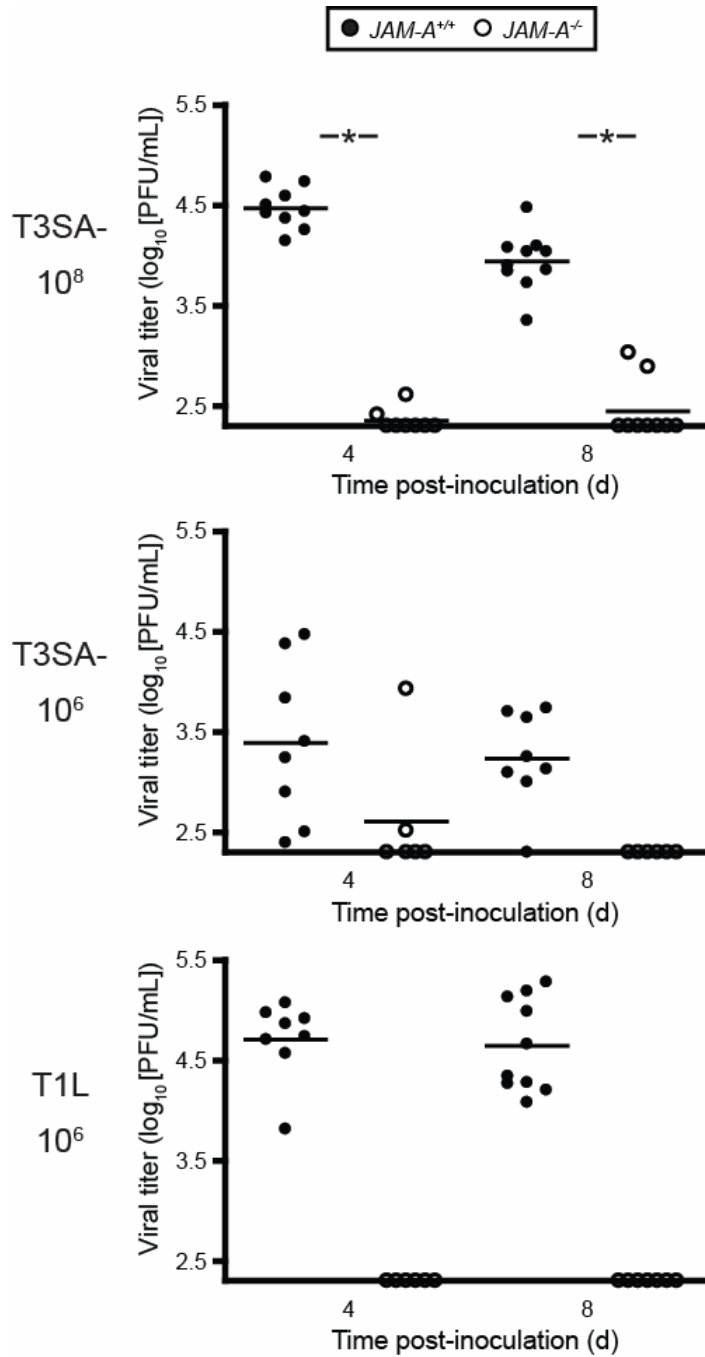


FIGURE IV-4. JAM-A is required for high-titer reovirus viremia. Newborn *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were inoculated perorally with 10⁸ PFU of T3SA- (top), 10⁶ PFU of T3SA- (middle), or 10⁶ PFU of T1L (bottom). At days 4 and 8, mice were euthanized, blood was collected, and viral titer in the blood was determined by plaque assay. Each data point represents one animal. Horizontal bars indicate the arithmetic mean of log-transformed data. *, *P* < 0.05 by Student's *t* test. When all values are less than the limit of detection (T3SA- 10⁶ day 8, T1L day 4 and day 8 in *JAM-A*^{-/-} mice), a Student's *t* test *P* value cannot be calculated.

JAM-A is required for efficient infection of primary endothelial cells – Endothelial cells (ECs) line blood vessels and serve as portals for virus entry into the circulation (90). To test whether JAM-A is required for reovirus infection of ECs, we infected primary mouse lung endothelial cells prepared from adult wild-type and *JAM-A*^{-/-} mice with T1L and T3SA-. The ECs displayed classic cobblestone morphology (Figure IV-5 A) and differed in JAM-A expression (Figure IV-5 B). Both strains of reovirus were capable of infecting wild-type but not *JAM-A*^{-/-} ECs over the course of a single infectious cycle (Figures IV-5 C and D). These results indicate that JAM-A expression is required for efficient infection of mouse ECs by reovirus.

Discussion

Reovirus strains disseminate systemically via hematogenous, neural, or a combination of both routes (44, 61, 85, 136). Spread of T3D from hindlimb to spinal cord, which occurs by neural routes (136), is not dependent on JAM-A. In contrast, spread of T1L from hindlimb to spinal cord, which occurs by hematogenous routes (136), is markedly diminished in *JAM-A*^{-/-} animals in comparison to wild-type controls. Additionally, primary neurons isolated from wild-type and *JAM-A*^{-/-} mice are equivalently susceptible to reovirus infection. These findings indicate that JAM-A mediates hematogenous but not neural dissemination of reovirus.

Viruses can establish viremia by several different mechanisms, including transport through lymphatics, infection of endothelial cells, and infection of blood leukocytes (90). However, mechanisms of reovirus viremia are unknown. Titers of T1L and T3SA- in the blood are diminished or undetectable in all *JAM-A*^{-/-} mice following peroral inoculation.

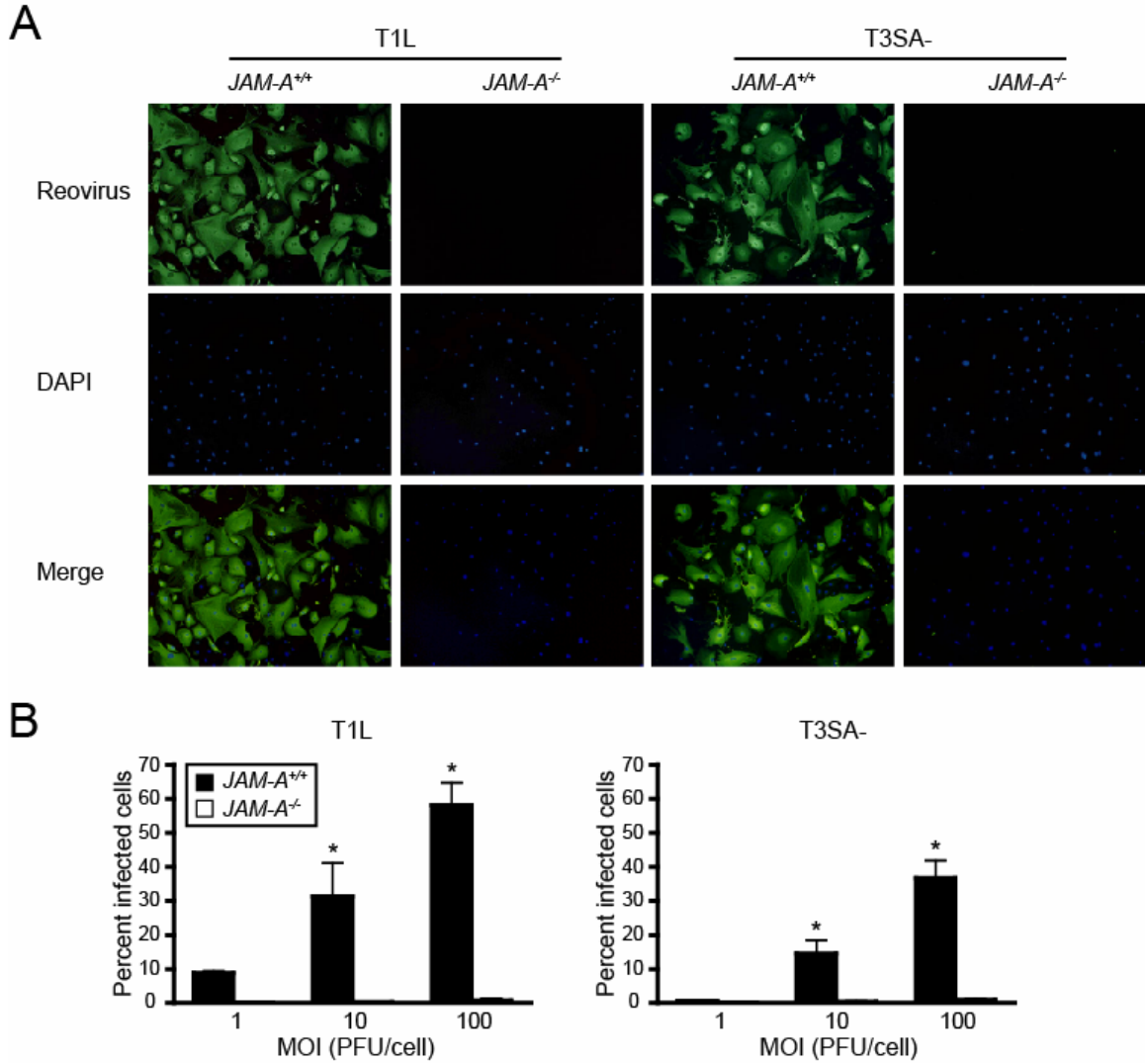


FIGURE IV-5. JAM-A is required for infection of primary endothelial cells. (A-B) Primary endothelial cells generated from *JAM-A*^{+/+} and *JAM-A*^{-/-} mice were adsorbed with either reovirus T1L or T3SA- at MOIs of 1, 10, and 100 PFU/cell and incubated for 20 hr. Cells were stained with polyclonal reovirus antiserum to detect reovirus antigen (green) and 4'6-diamidino-2-phenylindole (DAPI) to detect nuclei (blue) and visualized using indirect immunofluorescence microscopy. Representative fields of view from triplicate experiments using an MOI of 100 PFU/cell are shown (A). The percentage of infected cells was quantified by dividing the number of cells exhibiting reovirus staining by the total number of cell nuclei exhibiting DAPI staining in entire wells of 96-well plates for triplicate experiments (B). Wells contained between 200 and 1600 nuclei. Error bars indicate SD. *, *P* < 0.05 as determined by Student's *t* test in comparison to *JAM-A*^{-/-} endothelial cells at the same MOI.

Nevertheless, both strains of reovirus are capable of reaching the spleen in *JAM-A*^{-/-} mice following peroral inoculation, albeit in diminished titers in comparison to those in wild-type animals. Since the only hematogenous pathway from the intestine to the spleen is via the blood, the presence of virus in the spleen of *JAM-A*^{-/-} mice following peroral inoculation indicates that some virus likely escapes into the bloodstream in these animals, although viremic titer is reduced below the limit of detection in most *JAM-A*^{-/-} animals. Lymphatic spread of T1L from intestine to MLN is diminished but not abolished in *JAM-A*^{-/-} animals in comparison to wt animals. Most strikingly, JAM-A is required for infection of primary endothelial cells. Thus, two important routes to the establishment of viremia – spread through lymphatics and infection of endothelial cells - are impaired in *JAM-A*^{-/-} animals.

TJs of the intestinal mucosa of *JAM-A*^{-/-} mice demonstrate normal expression of occludin and claudin-2, upregulation of claudin-10 and claudin-15, and increased permeability to small molecules but not bacteria (70). Alterations in the ratios of claudin-10 and claudin-15 can affect the permeability of the epithelium to small molecules (130, 138), but the normal expression of TJ structural proteins such as occludin, zonula occludens-1, and E-cadherin (75) suggests that the absence of JAM-A does not lead to overt changes in TJ structure or assembly (70). Reovirus virions are ~ 25,000 times larger than the small molecules used in the permeability experiments reported previously (70) and roughly the size of a TJ. Therefore, I do not think the absence of JAM-A leads to the free flux of reovirus between intestinal epithelium and underlying tissue. This conclusion is supported by intestinal micrographs demonstrating that the absence of JAM-A does not alter the histologic pattern of reovirus intestinal infection. Moreover,

any alterations in the permeability of *JAM-A*^{-/-} intercellular junctions do not enhance the capacity of reovirus to access the lymphatic circulation and the bloodstream following replication in the intestine. Instead, my results indicate that specific interactions of reovirus with JAM-A promote hematogenous dissemination.

In this chapter, I report that JAM-A is not required for neural dissemination of T3D from the hindlimb but is required to establish reovirus viremia and to reach peak titers in lymphatic tissues. These results, together with those presented in Chapters II and III, indicate that JAM-A mediates hematogenous dissemination of reovirus.

CHAPTER V

SUMMARY AND FUTURE DIRECTIONS

Virus attachment to cells is mediated by discrete cellular receptors, many of which are members of the IgSF. JAM-A is one such IgSF molecule and is the only identified proteinaceous receptor for reovirus (4). Although the atomic details of reovirus-JAM-A interactions have been elucidated, little is known about how JAM-A mediates reovirus infection in vivo. The goal of my dissertation research was to determine the role of JAM-A in reovirus pathogenesis. Findings presented in this thesis provide evidence that JAM-A mediates hematogenous dissemination of reovirus. This chapter summarizes the findings presented herein, integrates the findings with what is understood about other IgSF virus receptors, and provides insights into future directions for this research.

Mechanisms of establishment of viremia

Viruses disseminate systemically either in the bloodstream, via nerves, or using a combination of both routes. Viremia is established through one or more sites, including efferent lymphatics, vascular endothelium, and peripheral blood leukocytes. Viruses that access the bloodstream via lymphatics do so by transiting from the site of entry through afferent lymphatic vessels, regional lymph nodes, efferent lymphatic vessels, and the thoracic duct, which connects the lymphatic system to the blood circulatory system. Viremia is often preceded by replication within regional lymph nodes, but passive

transport of virus from the entry site through lymphatics to the bloodstream can be achieved without viral replication within lymphatic nodes (90).

Several viruses access the bloodstream via the vascular endothelium. Infection of, transit between, and transcytosis through lymphatic and blood endothelial cells afford viral passage from infected tissue to the circulation. Infection of endothelial cells generates the majority of viral load found in the bloodstream 48 hours following infection by murine cytomegalovirus (112). Free transit between polarized cells by adenovirus is facilitated by interactions of its attachment protein, fiber, with its junction-associated receptor, CAR, disrupting junctional integrity (144). Penetration of endothelial cells via transcytosis efficiently transports adeno-associated virus (34) and HIV-1 (53) across endothelial cells. A final means of viral entry into the bloodstream is via infection of peripheral blood leukocytes. Colorado tick fever virus, a member of the *Reoviridae* family, replicates in erythroblasts in the bone marrow and disseminates systemically within erythrocytes (95). Despite knowledge of the possible mechanisms by which viruses gain access to the bloodstream, until this work, it was unclear how reovirus establishes viremia.

The role of JAM-A in reovirus dissemination

Data presented in Chapter II provide evidence that reovirus interactions with JAM-A are required for replication in target organs and production of encephalitis but surprisingly dispensable for intestinal replication following peroral inoculation. Failure of reovirus to cause encephalitis in *JAM-A*^{-/-} mice following peroral inoculation is not due to an intrinsic block to reovirus replication in *JAM-A*^{-/-} neural tissues. Intracranial

inoculation studies presented in Chapter III demonstrate that interactions of reovirus with JAM-A are not essential for reovirus encephalitis and replication in the murine CNS. Instead, expression of JAM-A is required for reovirus dissemination in the host.

Reovirus strains disseminate systemically via hematogenous, neural, or both routes (44, 61, 85, 136). Data presented in Chapter IV provide evidence that reovirus interactions with JAM-A are required for efficient hematogenous dissemination. In particular, lymphatic spread of T1L from intestine to MLN is diminished in *JAM-A*^{-/-} animals compared to wild-type animals. Furthermore, JAM-A is required for reovirus infection of primary endothelial cells. Thus, both spread through lymphatics and infection of endothelial cells are impaired in *JAM-A*^{-/-} animals.

How might JAM-A promote entry of reovirus into the lymph and bloodstream? I envision four possibilities. First, virus binding to JAM-A might lead to productive infection of lymphatic and blood endothelial cells with subsequent apical release of progeny virions into the circulation. In support of this idea, reovirus infection of polarized airway epithelial cells leads to apical release of virus with little detectable cytopathic effect (43). Second, JAM-A might be required for reovirus particles to traffic between endothelial cells, analogous to interactions of adenovirus with polarized cells. Although reovirus does not appear to disrupt epithelial tight junctions (43), it is possible that reovirus disrupts JAM-A interactions in endothelial tight junctions to permit bloodstream entry. Third, JAM-A might be required for reovirus transport through endothelial cells via a transcellular pathway. Caveolae-mediated transcytosis efficiently transports extracellular molecules across endothelial cells in a receptor-specific and cell type-specific manner (100). It is possible that reovirus transcytoses through endothelial

cells via a similar mechanism dependent on JAM-A. Fourth, JAM-A might be required for infection of peripheral blood leukocytes that transport reovirus in lymphatic and bloodstream circulation. Although reovirus infects both wild-type and *JAM-A*^{-/-} mononuclear cells of PPs, which comprise the gut-associated lymphatic tissue, the role of JAM-A in reovirus infection of leukocytes has not been determined.

Reovirus has been observed to be free in the plasma and associated with cells (60). Immunofluorescence and EM studies have demonstrated that viremic reovirus is observed in extracellular and macrophagic membrane-bound structures, cell-free aggregates, and leukocytes (60). Therefore, it is possible that reovirus establishes viremia through more than one mechanism. Results in this thesis indicate that JAM-A mediates a primary route of viremic access. It is conceivable that JAM-A-independent mechanisms account for a minority of viral bloodstream dissemination, accounting for the low levels of viremia in *JAM-A*^{-/-} mice.

Future directions

It is not known how JAM-A promotes lymphatic and circulatory access to reovirus in the infected host. Data presented in Chapter IV demonstrate that JAM-A is required for reovirus infection of nonpolarized primary endothelial cells, but this experiment probably does not mimic the conditions in which reovirus infects endothelial cells in vivo. To test whether JAM-A is required for reovirus infection of polarized endothelial cells, primary endothelial cells should be isolated from wild-type and *JAM-A*^{-/-} mice and plated in densities to promote polarization. T1 and T3 reovirus infection of these cells from the apical and basolateral surfaces should be compared. In addition, it

should be determined whether progeny virions are released from the apical or basolateral surface. These experiments will elucidate the role of JAM-A in reovirus infection of polarized endothelial cells. Immunohistochemical analysis of endothelial infection of intestine and target organs following peroral inoculation with reovirus will clarify whether endothelial cell infection occurs at sites from which virus accesses the bloodstream and at sites to which virus egresses from the bloodstream into target organ tissue. Furthermore, mice have been generated that do not express JAM-A in endothelial cells (19). Studies of reovirus pathogenesis similar to those presented in Chapters II and III using these mice will definitively determine whether JAM-A expression by endothelial cells is the primary determinant of hematogenous dissemination of reovirus.

It is possible that JAM-A is required for reovirus infection of blood leukocytes. Reovirus may be transported in the bloodstream by these cells, analogous to the related Colorado tick fever virus. Reoviruses replicate in splenic mononuclear cells of neonatal mice (128), and reovirus infects peripheral lymphatic tissues such as the spleen and PPs (45, 61, 85, 155). To test whether JAM-A is required for reovirus infection of leukocytes, splenocytes from neonatal wild-type and *JAM-A*^{-/-} mice should be isolated and infected with T1 and T3 reovirus strains. If *JAM-A*^{-/-} splenocytes demonstrate reduced capacity to support reovirus infection, splenocytes from these and wild-type animals should be flow sorted by cell type before infection with T1 and T3 reovirus strains. These experiments will clarify which leukocytic populations reovirus targets and which populations require JAM-A for infection. Collectively, these studies will contribute to a greater understanding of how reovirus establishes viremia and elucidate the cells types that require JAM-A for hematogenous dissemination of reovirus.

The role of IgSF receptors in viral pathogenesis

Diverse families of viruses bind immunoglobulin superfamily (IgSF) proteins located in TJs and adherens junctions of epithelium and endothelium. However, little is known about the roles of these receptors in the pathogenesis of viral disease. In addition to the work presented here, there are only two other studies that have explored functions of IgSF virus receptors in viral pathogenesis: nectin-1 and herpes simplex virus-2 (HSV-2) infection of mice and carcinoembryonic antigen-related cell adhesion molecule 1a (CEACAM1a) and mouse hepatitis virus (MHV) infection of mice.

HSV uses three cell-surface molecules to mediate entry into cells: herpesvirus entry mediator (HVEM), nectin-1, and nectin-2 (27, 49, 84, 145). Nectins, like JAMs, are IgSF molecules involved in cell adhesion (127). Nectin-1, also called poliovirus receptor-related 1 (PVRL1), is widely expressed and found in neural, epithelial, stromal, endothelial, and lymphoid cells (54). As with reovirus and JAM-A, absence of nectin-1 attenuates HSV-2 virulence in intravaginally inoculated *Pvr11*^{-/-} mice. Unlike JAM-A and reovirus, *Pvr11*^{-/-} mice exhibit reduced efficiency of infection by HSV-2 at the site of primary replication, the vaginal epithelium. Replication at this site requires either nectin-1 or HVEM, which is a member of the tumor necrosis factor receptor family. In addition, spread of HSV-2 to the dorsal root ganglia and spinal cord is impaired in these animals. Although a subset of *Pvr11*^{-/-} mice succumb to HSV infection, these animals are protected from development of external signs of disease, including hair loss, inflammation, and skin lesions, indicating that nectin-1 mediates spread from vaginal epithelium to perineal skin and surrounding areas (129). In contrast to mice lacking nectin-1, mice lacking HVEM do not exhibit alterations in HSV-2 vaginal infection, external lesions, or viral

loads in the peripheral nervous system or CNS. Thus, IgSF member nectin-1 mediates HSV-2 infection in the vaginal epithelium, spread to perineal skin, and dissemination to the CNS.

CEACAM1a serves as a receptor for the coronavirus MHV and also binds several bacteria, including *Escherichia coli*, *Neisseria gonorrhoeae*, *N. meningitidis*, and *Haemophilus influenzae*. CEACAM1a is a glycosylated protein expressed as four isoforms with either two or four Ig domains. CEACAM1a is widely expressed on epithelial cells, is found on various leukocytes, is expressed at low levels on glial cells in the nervous system, and is inducible on endothelial cells and T cells (69). *Ceacam1a*^{-/-} mice are completely resistant to intranasal and intracerebral inoculation of MHV strain A59 (58). However, *Ceacam1a*^{-/-} mice will succumb to a more neurovirulent strain of MHV, strain JHM, at inoculation doses 100-fold higher than those required for morbidity in wild-type mice (83). The capacity of MHV-JHM to kill *Ceacam1a*^{-/-} mice is either due to alternative receptor utilization by this viral strain or a receptor-independent means of spread. Receptor-independent spread of MHV occurs when the viral spike glycoprotein, which mediates viral attachment and fusion, is expressed at the surface of infected cells and induces fusion of infected cells with uninfected cells to promote direct cell-to-cell spread of virus (83). Thus, although CEACAM1a promotes efficient replication of MHV strains in vivo, the requirement for CEACAM1a expression for MHV disease is strain-specific.

CEACAM1a is required for infection by MHV A59 at all sites. In contrast, expression of JAM-A and nectin-1 are not absolute requirements for viral replication of reovirus in the intestine and HSV-2 in the vaginal epithelium, respectively. In the case of

HSV-2, HVEM contributes to vaginal epithelial infection. In the case of reovirus, a virus receptor other than JAM-A likely mediates infection in the intestine. Strikingly, the IgSF molecules JAM-A and nectin-1 both mediate spread of virus from the site of primary replication to target organs. These observations raise the possibility of a conserved mechanism of dissemination specific to the interactions of viruses with their IgSF receptors.

Evidence for currently-unknown reovirus receptors

The exquisite neural tropism of reovirus in newborn mice is restricted to T3 strains (65, 78, 102). T3SA- does not bind sialic acid (3), a carbohydrate coreceptor used by some T3 reovirus strains (32). That JAM-A is not required for reovirus T3SA- replication in the intestine or brain suggests the existence of novel receptors for reovirus at those sites. In parallel with these findings, analysis of histologic brain sections indicates that T3SA- infects the same regions of wild-type and *JAM-A*^{-/-} mouse brains: the cortex, CA2-4 regions of the hippocampus, thalamus, and cerebellar Purkinje cells. This regional tropism is identical to that observed using sialic acid-binding T3 reovirus strains (31, 105). Moreover, genetic deletion of JAM-A does not alter reovirus infection of primary cultures of neurons. Based on findings reported here, I hypothesize that reovirus replication in neurons is mediated by a serotype-specific receptor other than JAM-A or sialic acid. In addition, since both T1 and T3 reovirus strains can replicate in the intestines of *JAM-A*^{-/-} mice, I propose that reovirus infection at that site is mediated by a serotype-independent receptor or a receptor-independent uptake mechanism.

To identify receptors that mediate reovirus infection of neurons, proteomic, genomic, and bioinformatic approaches should be used. A virus-on-protein-binding assay (VOPBA) utilizing radiolabeled reovirus particles and membrane protein preparations from *JAM-A*^{-/-} primary murine cortical cultures, combined with protein identification by mass spectrometry, should be used as a first step to identify candidate receptors. A screening approach using existing neural cDNA libraries or a cDNA library generated from *JAM-A*^{-/-} primary cortical neurons can also be used to identify a neural-specific reovirus receptor. Utilizing knowledge of reovirus tropism in the brain generated in this thesis and reported in the literature (133), a database of gene-expression patterns in the mouse brain such as the gene expression nervous system atlas (GENSAT) database (159) can be searched for receptor-like proteins expressed in reovirus-targeted regions while excluding those expressed in regions refractory to reovirus infection, generating a pool of potential reovirus receptors.

Conclusion

The finding that an individual viral receptor can mediate a specific step in pathogenesis has important implications for antiviral strategies. For example, while genetic deletion of JAM-A protects mice from reovirus morbidity and mortality following peroral inoculation, these animals are capable of viral transmission to new hosts. Uninoculated littermates of infected *JAM-A*^{-/-} mice develop high titers of reovirus in the intestine, suggesting that viral shedding occurs in infected *JAM-A*^{-/-} mice (Appendix A). In this viral model, pharmacological blockade of reovirus-JAM-A interactions would diminish disease in treated individuals but have no effect on viral

transmission between hosts, an observation that merits consideration in other viral diseases.

Establishment of viremia is a poorly understood process for most viruses. My work shows that the establishment of reovirus viremia depends on virus interactions with JAM-A. Further dissection of how JAM-A mediates viremic access may provide clues about mechanisms of systemic dissemination of adenovirus, coxsackievirus, herpes simplex virus, and poliovirus, which also employ junction-associated IgSF members as receptors. Moreover, our finding that a broadly-expressed receptor mediates an exquisitely specific aspect of viral pathogenesis suggests that virus-host interactions require multiple receptors that serve unique functions at each step of the disease process.

CHAPTER VI

MATERIALS AND METHODS

Cell lines, viruses, and antibodies

L cells were maintained as described (3). Reovirus strains T1L and T3D are laboratory stocks. T3/C44-SA- (T3SA-) was generated as described (3). Virus was purified after growth in L cells by CsCl-gradient centrifugation (48). Viral titers were determined either by plaque assay (141) or fluorescent focus assay (3). Immunoglobulin G (IgG) fractions of rabbit antisera raised against T1L and T3D (151) were purified by protein A-Sepharose (3). Fluorescently conjugated secondary Alexa antibodies were obtained from Molecular Probes (Invitrogen). Murine mAbs 9BG5 (17) and 5C6 (142) were purified from hybridoma supernatants (Cell Culture Center, Minneapolis, MN).

Mice

C57BL/6J (wild-type, *JAM-A*^{+/+}) were obtained from Jackson Laboratory. *JAM-A*^{-/-} mice (19) were provided by T. Sato (Cornell University, New York, NY) and backcrossed for ten generations on a C57BL/6J background strain. Disruption of the *JAM-A* gene was confirmed by PCR.

Primary cells

MEFs were maintained in Dulbecco's modified Eagle's medium (Gibco) supplemented to contain 10% FBS (Gibco), 1x MEM non-essential amino acids, 2 mM

L-glutamine, 0.1 mM 2-mercaptoethanol, 20mM HEPES, 100 units/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. Experiments were performed using MEFs following the third to fifth passage.

Primary mouse cortical cultures were derived from cortices of E15 wild-type and *JAM-A*^{-/-} embryos. Fetuses were decapitated, brains were removed, and cortical lobes were dissected and submerged in Hanks' balanced salt solution (Gibco) on ice. Cortices were incubated in 0.6 mg/ml trypsin solution at RT for 30 min, washed twice, and then manually dissociated twice with a pasteur pipette. Settled aggregates were discarded. Viable cells, quantified by trypan blue staining, were plated at a density of 2.75 x 10⁵ cells/ml in 24-well plates (Costar) or on glass coverslips placed in 24-well plates (BD). Wells were treated prior to plating with a 10 µg/ml poly-D-lysine solution (BD) and a 1.64 µg/ml laminin solution (BD). Cultures were incubated for the first 24 h in neurobasal media (Gibco) supplemented to contain 10% FBS (Gibco), 0.6 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cultures were thereafter maintained in neurobasal media supplemented to contain 10 ml 50X B27 (Gibco), 50 units/ml penicillin, and 50 µg/ml streptomycin. A 50% medium change was performed every 3-4 days. Neurons were allowed to mature for 7 days prior to use.

Primary mouse lung endothelial cells were derived from two- to four-month old wild-type and *JAM-A*^{-/-} mice. Mice were euthanized and the lung vasculature was perfused via the right ventricle with PBS supplemented to contain 2 mM EDTA followed by 0.25% trypsin (Invitrogen) supplemented to contain 2 mM EDTA. Heart and lungs were removed en bloc and incubated at 37°C for 20 min. The visceral pleura was trimmed away, and the perfusion was repeated. Primary endothelial cells were recovered

and grown on tissue culture plastic for 12 days in EGM-2 Basal Medium (Clonetics) supplemented to contain EGM-2 MV SingleQuot (Clonetics) prior to use.

Viral infectivity

Monolayers of cells were adsorbed with reovirus at various MOIs, fixed after 20 h, stained, visualized by indirect immunofluorescence, and quantified as described (3). When indicated, *A. ureafaciens* neuraminidase (Sigma) was applied to cells at 37°C for 60 min prior to infection with reovirus. When indicated, 9BG5 and 5C6 were added to virus preparations before infection of cells.

Virus replication

Monolayers of cells in 24-well plates (Costar) were adsorbed with reovirus at an MOI of 2 PFU/cell, washed with PBS, and incubated for various intervals. Cells were frozen and thawed twice prior to viral titer determination by plaque assay using L cells. Viral yields were calculated according to the following formula: $\log_{10} \text{yield}_{tx} = \log_{10} (\text{PFU/ml})_{tx} - \log_{10} (\text{PFU/ml})_{t0}$, where tx is the time post infection.

Infection of mice

Two- and three-day old mice weighing 1.5-2 grams were inoculated perorally (108), intracranially (135), or intramuscularly with purified reovirus diluted in PBS. For analysis of virulence, mice were monitored for weight loss and symptoms of disease for 21 days post-inoculation and euthanized when found to be moribund. For analysis of viral replication, mice were euthanized at various intervals following inoculation, and

organs were harvested into 1 ml of PBS, frozen and thawed three times, and homogenized by sonication. For analysis of viremia, mice were decapitated at various intervals following inoculation, and whole blood was collected from the neck into a 1 ml syringe containing 100 μ l Alsever's solution (Sigma). Blood in Alsever's solution was frozen and thawed three times and homogenized by sonication. Viral titers in organ homogenates and blood were determined by plaque assay. Animal husbandry and experimental procedures were performed in accordance with Public Health Service policy and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Histology

Two- and three-day old mice weighing 1.5-2 grams were inoculated intracranially or perorally with purified reovirus diluted in PBS. At various intervals following inoculation, mice were euthanized and organs were collected. Brains were bisected sagittally and the right hemispheres were processed for histopathologic analysis. Peyer's patches and surrounding tissue were collected from the small intestine and processed for histopathologic analysis. Tissues were incubated in 10% formalin at RT for 24 h followed by incubation in 70% ethanol at RT. Fixed organs were embedded in paraffin, and 6- μ m coronal sections were prepared. Consecutively obtained sections were stained with H&E for evaluation of histopathologic changes or processed for immunohistochemical detection of reovirus protein.

APPENDIX A

JAM-A IS NOT REQUIRED FOR TRANSMISSION OF REOVIRUS
BETWEEN LITTERMATES

Introduction

Reovirus strains are capable of transmission from perorally infected newborn mice to naïve littermates. T1L and T3 clone 9 are successfully transmitted to uninoculated littermates a majority of the time, whereas T3D is rarely transmitted to littermates (63). This capacity for reovirus transmission between hosts segregates with the L2 gene segment (63). L2 encodes $\lambda 2$, a viral protein that forms pentamers from which $\sigma 1$ extends and through which viral messenger RNA extrudes during transcription (114).

This thesis presents results indicating that JAM-A is required for dissemination of reovirus within the host. However, it is not known whether expression of JAM-A is required for transmission of reovirus between hosts. In this study, I used wild-type and *JAM-A*^{-/-} mice to determine the extent to which JAM-A is required for transmission of reovirus among littermates. I found that JAM-A is not required for animal-to-animal transmission of reovirus.

Results

To determine the function of JAM-A in reovirus transmission between littermates, I selected two newborn wild-type and *JAM-A*^{-/-} mice from litters of eight for peroral inoculation with 10^4 PFU of T3SA-. I isolated the role of JAM-A from the role of sialic acid in reovirus transmission by using T3SA-, a reovirus strain incapable of binding sialic acid (3). The remaining six mice in each litter received no treatment. Litters were reunited immediately following inoculation. I observed and marked infected mice daily and quantified viral titers in various organs of all mice twelve days post-inoculation.

T3SA- replication was observed in the intestines of all inoculated wild-type and *JAM-A*^{-/-} mice (Figure 1). All uninoculated *JAM-A*^{-/-} littermates displayed robust intestinal replication, whereas only two of six uninoculated wild-type littermates harbored viral titer in the intestines (Figure 1). These results indicate that JAM-A is not required for transmission of reovirus between littermates. Analogous to results presented in Chapter II, replication in the brain of inoculated wild-type mice is much greater than that seen in inoculated *JAM-A*^{-/-} mice (Figure 1). However, no viral replication in the brain was observed in any of the uninoculated *JAM-A*^{-/-} mice and in all except one of the uninoculated wild-type mice (Figure 1). These results confirm that, even though reovirus is transmitted between *JAM-A*^{-/-} mice, all *JAM-A*^{-/-} mice are protected from disease.

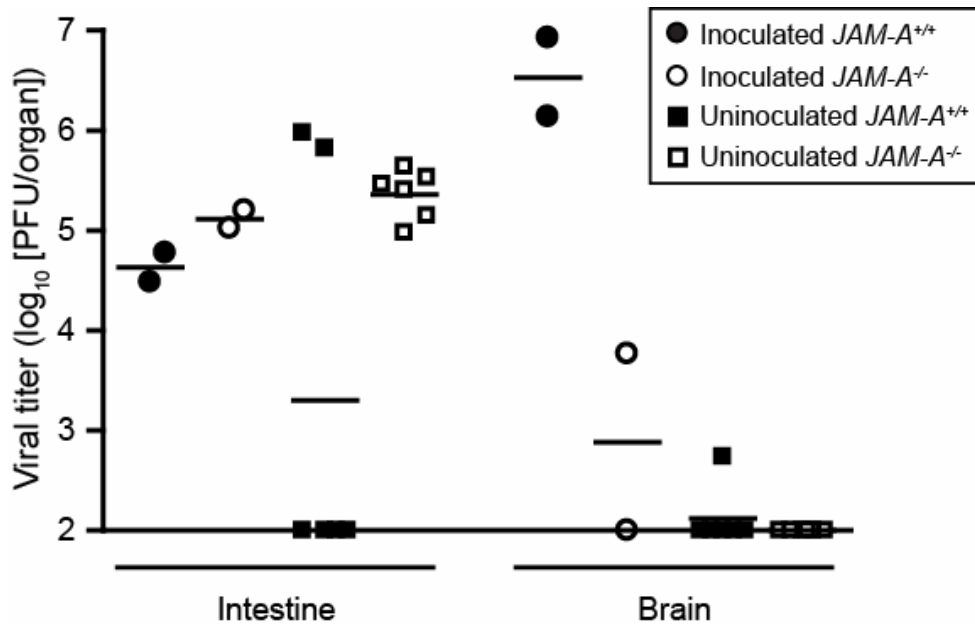


FIGURE 1. Transmission of reovirus between littermates is not dependent on JAM-A. Two newborn *JAM-A*^{+/+} and two newborn *JAM-A*^{-/-} mice from litters of eight were inoculated perorally with 10⁴ PFU T3SA- and placed back with uninfected littermates. At day 12 after inoculation, mice were euthanized, intestines and brains were resected, and viral titers were determined by plaque assay. Each data point represents one animal. Horizontal bars indicate the arithmetic mean of log-transformed data.

Discussion

I have demonstrated in Chapter II that T3SA- viral replication in the intestine of wild-type and *JAM-A*^{-/-} mice is equivalent. In Chapter IV, I present histologic evidence indicating that no substantial differences in reovirus intestinal tropism exist between wild-type and *JAM-A*^{-/-} mice. Moreover, transmission of reovirus between hosts has been shown to segregate with the viral L2 gene (63). Therefore, it is not surprising that work presented in this section demonstrates that JAM-A expression is not required for reovirus transmission between hosts.

As discussed in Chapter V, work presented in this thesis suggests the existence of an unidentified reovirus intestinal receptor. I hypothesize that reovirus engagement of this receptor is required for viral replication in the intestine and also for transmission between hosts. It has not been definitively shown that the reovirus L2 gene is important for viral replication in the intestine, but it is possible that this gene mediates engagement of an intestinal receptor, release from the intestinal cells into the intestinal lumen, or stability of reovirus in the environment (12, 63). Nonetheless, this experiment demonstrates that the role of JAM-A in reovirus pathogenesis is limited to viral dissemination within - but not between - hosts.

APPENDIX B

A PLASMID-BASED REVERSE GENETICS SYSTEM FOR ANIMAL DOUBLE-STRANDED RNA VIRUSES

Takeshi Kobayashi, Annukka A. R. Antar, Karl W. Boehme, Pranav Danthi, Elizabeth A.

Eby, Kristen M. Guglielmi, Geoffrey H. Holm, Elizabeth M. Johnson, Melissa S.

Maginnis, Sam Naik, Wesley B. Skelton, J. Denise Wetzell, Gregory J. Wilson, James D.

Chappell, and Terence S. Dermody

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A Plasmid-Based Reverse Genetics System for Animal Double-Stranded RNA Viruses

Takeshi Kobayashi,^{1,2} Annukka A.R. Antar,^{2,3,5} Karl W. Boehme,^{1,2,5} Pranav Danthi,^{1,2,5} Elizabeth A. Eby,^{2,3,5} Kristen M. Guglielmi,^{2,3,5} Geoffrey H. Holm,^{1,2,5} Elizabeth M. Johnson,^{2,3,5} Melissa S. Maginnis,^{2,3,5} Sam Naik,^{2,4,5} Wesley B. Skelton,^{1,2,5} J. Denise Wetzel,^{1,2,5} Gregory J. Wilson,^{1,2,5} James D. Chappell,^{1,2,4,*} and Terence S. Dermody^{1,2,3,*}

¹Department of Pediatrics

²Elizabeth B. Lamb Center for Pediatric Research

³Department of Microbiology and Immunology

⁴Department of Pathology

Vanderbilt University School of Medicine, Nashville, TN 37232, USA

⁵These authors contributed equally to this work.

*Correspondence: jim.chappell@vanderbilt.edu (J.D.C.), terry.dermody@vanderbilt.edu (T.S.D.)

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SUMMARY

Mammalian orthoreoviruses (reoviruses) are highly tractable experimental models for studies of double-stranded (ds) RNA virus replication and pathogenesis. Reoviruses infect respiratory and intestinal epithelium and disseminate systemically in newborn animals. Until now, a strategy to rescue infectious virus from cloned cDNA has not been available for any member of the *Reoviridae* family of dsRNA viruses. We report the generation of viable reovirus following plasmid transfection of murine L929 (L) cells using a strategy free of helper virus and independent of selection. We used the reovirus reverse genetics system to introduce mutations into viral capsid proteins $\sigma 1$ and $\sigma 3$ and to rescue a virus that expresses a green fluorescent protein (GFP) transgene, thus demonstrating the tractability of this technology. The plasmid-based reverse genetics approach described here can be exploited for studies of reovirus replication and pathogenesis and used to develop reovirus as a vaccine vector.

INTRODUCTION

Reoviruses are members of the *Reoviridae* family, which includes several genera that cause disease in humans and animals. Chief among these are the rotaviruses, which are the most common cause of viral gastroenteritis in human infants (Kapikian et al., 2001). Reoviruses infect the respiratory and gastrointestinal tracts of virtually all mammals, including humans (Tyler, 2001). However, disease associated with reovirus infection occurs primarily in the very young (Tyler et al., 2004). Reoviruses are highly virulent in newborn mice, the preferred experimental system for studies of reovirus pathogenesis, and produce injury

to a variety of host tissues, including the central nervous system (CNS), heart, and liver (Tyler, 2001).

Reoviruses contain a genome of 10 dsRNA gene segments enclosed in two concentric protein shells, termed *outer capsid* and *core*. Reovirus infection is initiated by viral attachment to host cells via the filamentous attachment protein $\sigma 1$ (Furlong et al., 1988). The $\sigma 1$ protein engages cell-surface carbohydrate (Chappell et al., 1997, 2000) and junctional adhesion molecule-A (JAM-A) (Barton et al., 2001b; Campbell et al., 2005), an integral component of intercellular tight junctions (Martin-Padura et al., 1998). Following attachment to the cell surface, reovirus internalization is mediated by $\beta 1$ integrins (Maginnis et al., 2006), most likely via clathrin-dependent endocytosis (Ehrlich et al., 2004). In the endocytic compartment, viral outer-capsid proteins $\sigma 3$ and $\mu 1/\mu 1C$ are cleaved by acid-dependent cysteine proteases (Baer and Dermody, 1997; Ebert et al., 2002), resulting in generation of infectious subviral particles (ISVPs) (Borsa et al., 1981). During ISVP formation, $\sigma 3$ is removed and a hydrophobic conformer of $\mu 1/\mu 1C$ is exposed, facilitating endosomal membrane penetration and delivery of transcriptionally active reovirus core particles into the cytoplasm (Chandran et al., 2002; Odegard et al., 2004), where the remainder of the replication cycle occurs.

With the exception of dsRNA viruses, a plasmid-based reverse genetics system exists for all major groups of animal RNA viruses, including bornaviruses, bunyaviruses, coronaviruses, flaviviruses, orthomyxoviruses, paramyxoviruses, picornaviruses, and rhabdoviruses (Table S1 in the Supplemental Data available with this article online). Despite extensive efforts in several laboratories, generation of an animal dsRNA virus entirely from cloned cDNAs has not been achieved. This critical technological gap is perhaps the single most important limitation to studies of these viruses. Previous efforts on reovirus and rotavirus reverse genetics have resulted in entirely RNA-based (Roner et al., 1997) or partially plasmid-based (Komoto et al., 2006) systems that permit replacement of one or two viral genes. These approaches have been used to

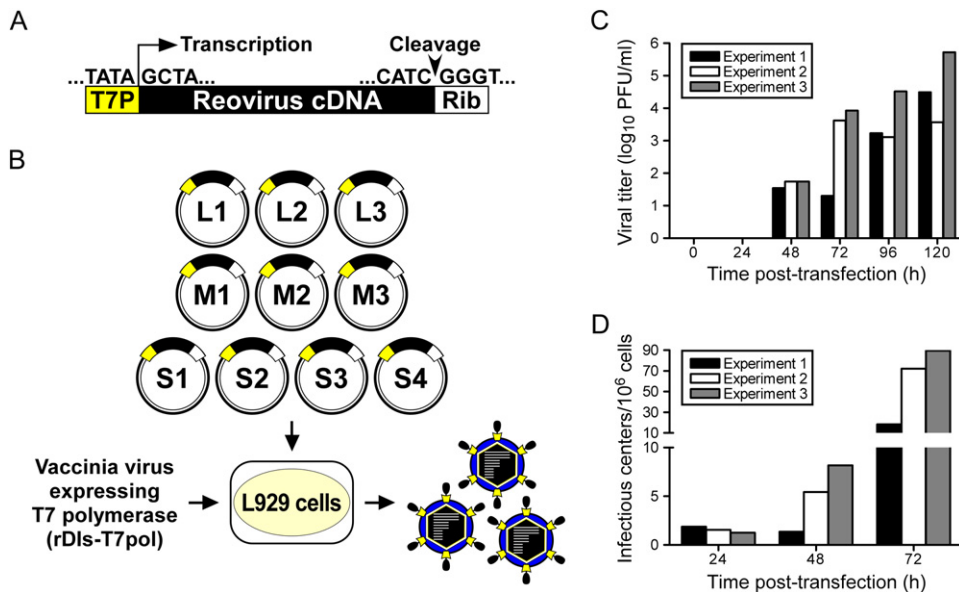


Figure 1. Experimental Strategy to Generate Reovirus from Cloned cDNA

(A) Prototype reovirus gene segment cDNA in plasmid. Cloned cDNAs representing each of the ten full-length reovirus RNA gene segments are flanked by the bacteriophage T7 RNA polymerase promoter (T7P) and the antigenomic hepatitis delta virus (HDV) ribozyme (Rib).

(B) Schematic of approach. The ten reovirus cDNA constructs are transfected into murine L cells expressing T7 RNA polymerase from recombinant vaccinia virus strain rDIs-T7pol, which is replication defective. Nascent transcripts correspond to viral mRNAs containing the native 5' end. Self cleavage by the HDV ribozyme generates the native viral 3' end. Following 5 days of incubation, transfected cells are lysed by freeze-thaw, and viable viruses rescued from cloned cDNAs are isolated by plaque assay using L cells.

(C) Kinetics of virus production following plasmid transfection of L cells. Cells were transfected with plasmid DNA according to the protocol in (B) and lysed at the intervals shown. Viral titers in cell lysates were determined by plaque assay.

(D) Infectious center assay following plasmid transfection of L cells. Cells were transfected with plasmid DNA, trypsinized at the intervals shown post-transfection, washed, counted, diluted, and applied directly to monolayers of untreated L cells. The number of the infectious centers was determined by plaque assay.

rescue temperature-sensitive reovirus strains (Roner et al., 1997), define packaging signals in reovirus RNAs (Roner and Steele, 2007), and isolate rotaviruses containing engineered changes in the viral attachment protein (Komoto et al., 2006). However, neither the reovirus nor rotavirus reverse genetics systems in their current configurations permit selective introduction and recovery of desired mutations in each viral gene segment.

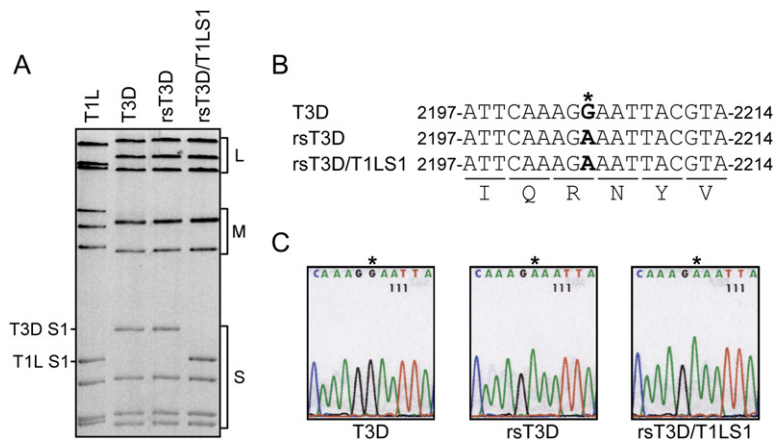
We report the development of an entirely plasmid-based reverse genetics system for mammalian reovirus in which viable viruses are generated from cloned cDNAs. Neither helper virus nor coexpression of viral replication proteins is required for recovery of wild-type (WT) virus or engineered viral mutants. Point mutations introduced into viral capsid proteins $\sigma 1$ and $\sigma 3$ were used to define sequences that govern susceptibility to cleavage by intestinal proteases. We also recovered a recombinant virus that expresses green fluorescent protein (GFP) by replacement of the $\sigma 3$ open reading frame (ORF) with GFP. The establishment of plasmid-based reverse genetics for reovirus will allow heretofore technically unapproachable problems in dsRNA virus biology to be studied, provide a platform for development of analogous marker rescue systems for other segmented dsRNA viruses, and foster exploration of reovirus as a vaccine

vector to elicit protective immunity against a variety of mucosal pathogens.

RESULTS

Generation of Viable Reovirus from Cloned cDNA

To generate recombinant reovirus from cloned cDNAs, plasmids encoding each of the ten viral gene segments were engineered to facilitate transcription of full-length viral mRNAs under control of the bacteriophage T7 RNA polymerase promoter, which directs transcription initiation preferentially from a juxtaposed guanosine residue (Milligan et al., 1987). As all reovirus (+)-sense RNAs are terminated with a 5' guanosine (Furuichi et al., 1975a, 1975b), plasmid-generated transcripts are anticipated to possess native 5' ends (Roner and Joklik, 2001) (Figure 1A). Murine L929 fibroblast (L) cells, which efficiently support reovirus replication (Barton et al., 2001a), were infected with the attenuated, T7 RNA polymerase-expressing vaccinia virus strain rDIs-T7pol 1 hr prior to transfection with the ten reovirus cDNA plasmids (Figure 1B). Nascent transcripts were synthesized with the hepatitis delta virus (HDV) ribozyme fused to the 3' terminus, which is expected to generate a native 3' end upon autocatalytic removal (Roner and Joklik, 2001) (Figure 1A). Thus, this



of the L1 gene was amplified by one-step RT-PCR performed using viral dsRNA extracted from purified virions of T3D, rsT3D, and rsT3D/T1LS1. Products were subjected to direct sequence analysis and compared to the L1 sequence of T3D. Shown are sequence chromatograms demonstrating G→A substitution at position 2205 of the rsT3D and rsT3D/T1LS1 L1 genes.

Figure 2. Rescue of rsT3D and rsT3D/T1LS1

(A) Electropherotypes of T1L, T3D, rsT3D, and rsT3D/T1LS1. Viral dsRNA was extracted from purified virions and electrophoresed in an SDS-polyacrylamide gel, followed by ethidium bromide staining to visualize viral gene segments. Size classes of gene segments (L, M, S) are indicated.

(B) Recombinant viruses contain a novel mutation in the L1 gene. The single nucleotide difference in L1 unique to rsT3D and rsT3D/T1LS1 is shown in the alignment as an asterisk. The G→A substitution at position 2205 is a signature change engineered into the cloned T3D L1 cDNA used for marker rescue.

(C) Sequence analysis of L1 gene RT-PCR products from rescued reoviruses. A fragment

expression strategy should yield ten unique reovirus mRNA species competent to complete all steps in the viral RNA life cycle. Accordingly, rescued viruses were recovered from cell-culture supernatants by plaque assay on L cell monolayers (Figure 1C).

To ensure that viruses isolated following plasmid transfection represented single clones, and to preclude contamination of reovirus stocks by rDIs-T7pol, all viruses were isolated by plaque purification using L cell monolayers. rDIs-T7pol is replication defective and produces no detectable growth in mammalian cells (Ishii et al., 2002). Concordantly, no viral plaques arose on L cell monolayers when the cotransfections were prepared with nine of the ten reovirus marker-rescue plasmids (data not shown). Furthermore, vaccinia virus proteins were not detected by immunoblot analysis of second-passage stocks of plaque-purified reoviruses (data not shown).

Infectious center assays were performed to assess the efficiency of reovirus infection in plasmid-transfected L cells. At 24–48 hr posttransfection, times corresponding to the primary round of infection, eight or fewer infectious centers per 10^6 cells were detected (Figure 1D). The number of infectious centers increased substantially between 48 and 72 hr posttransfection to 18 to 90 per 10^6 cells, suggesting that a secondary round of infection had ensued by the 72 hr time point. Viral titer in transfection lysates was below the limit of detection (~ 10 PFU/ml) at 24 hr but was detectable at 48 hr and increased logarithmically at time points thereafter (Figure 1C). The ratio of viral PFU in transfection lysates to infectious centers was ~ 10 to 100 at 48 and 72 hr. These results indicate that initiation of productive reovirus replication from transfected plasmids is inefficient, with approximately 1 cell per 10^5 to 10^6 cells giving rise to, on average, 10 to 100 viable virus particles that establish infection of the culture. In our experiments, yields of WT virus 5 days after plasmid transfection are regularly in the range of 10^4 – 10^6 PFU/ml (Figure 1C).

Separation of reovirus genomic dsRNA using SDS-PAGE produces unique electrophoretic patterns that can be used to discriminate different viral strains (Barton et al., 2001a). To confirm that viruses isolated using the plasmid-based rescue procedure contained the expected combination of gene segments, genomic dsRNA isolated from recombinant strain (rs) T3D and rsT3D/T1LS1 was resolved in SDS-polyacrylamide gels and visualized by ethidium bromide staining (Figure 2A). The electropherotype of rsT3D was indistinguishable from that of strain T3D, the origin of the cloned cDNA sequences used to generate rsT3D. Likewise, rsT3D/T1LS1 displayed an electropherotype consistent with nine cloned gene segments derived from T3D and a single cloned gene segment, S1, derived from strain T1L. To exclude the possibility of contamination, a silent point mutation, G to A at nucleotide 2205, was introduced into the L1 gene of all virus strains generated from cloned cDNAs (Figure 2B). This change has not been observed in any reported T3D L1 sequence (Wiener and Joklik, 1989) and serves as a signature for viruses derived through plasmid-based rescue. As anticipated, sequence analysis of rsT3D and rsT3D/T1LS1 revealed the expected G to A substitution (Figure 2C), confirming the plasmid origins of these isolates.

Characterization of Reoviruses Generated by Plasmid Transfection

Reoviruses replicate and assemble within cytoplasmic inclusions in infected cells (Fields, 1971). Viral inclusions contain dsRNA (Silverstein and Schur, 1970), viral proteins (Fields, 1971), and both complete and incomplete viral particles (Fields, 1971). Reovirus strain T3D forms large globular inclusions that localize to the perinuclear space (Parker et al., 2002). To determine whether rsT3D forms viral inclusions in a manner similar to native T3D, cells were infected with T3D and rsT3D and processed 18 hr postinfection for image analysis by confocal microscopy (Figure 3A). Both T3D and rsT3D formed morphologically indistinguishable large globular inclusions that were

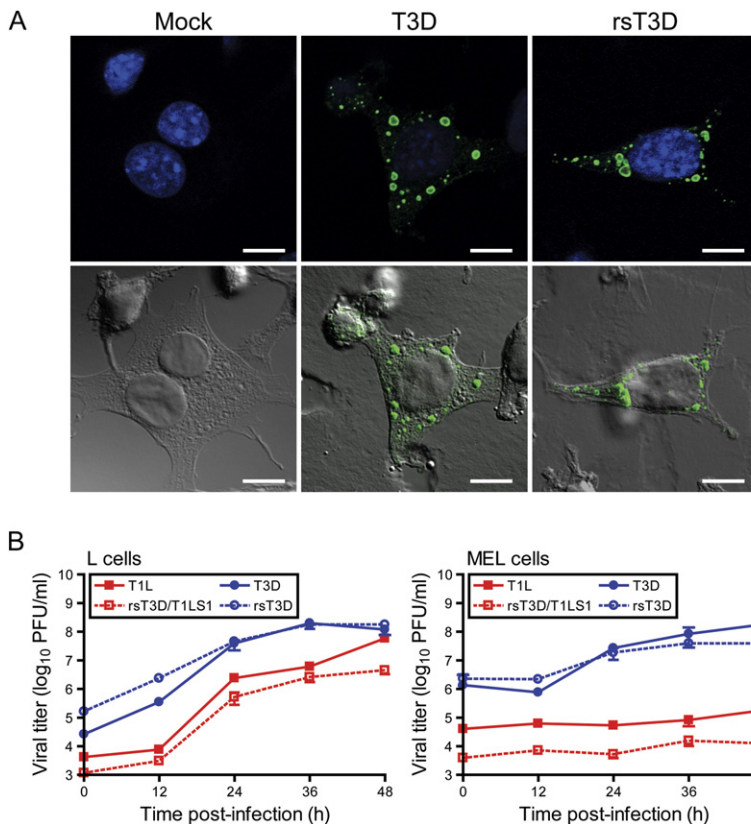


Figure 3. Infectivity of Native and Recombinant Reoviruses

(A) Immunofluorescence of cells infected with T3D and rsT3D. L cells were mock infected or infected with either T3D or rsT3D, stained at 18 hr postinfection with anti- μ NS antiserum to visualize reovirus inclusions (green) and TO-PRO3 to visualize nuclei (blue), and imaged by confocal laser scanning microscopy. Representative digital fluorescence (top panel) and DIC images (bottom panel) for mock-, T3D-, and rsT3D-infected cells are shown. Scale bar, 10 μ M.

(B) One-step growth curve for T1L, T3D, rsT3D, and rsT3D/T1LS1 in L cells (left) and MEL cells (right). Cells were infected with virus, incubated for the intervals shown, and lysed by freeze-thaw. Viral titers in cell lysates were determined by plaque assay. The results are presented as the mean viral titers for triplicate experiments. Error bars indicate SD.

localized to the perinuclear compartment. We conclude that recombinant rsT3D recapitulates a hallmark of native T3D infection in cultured cells.

To confirm that the recombinant viruses exhibit replication kinetics similar to the native strains, T1L, T3D, rsT3D, and rsT3D/T1LS1 were tested for infection of L cells and MEL cells (Figure 3B). L cells support replication of all reovirus strains tested in our laboratory. In contrast, MEL cells support replication of only sialic acid-binding reovirus strains (Rubin et al., 1992; Chappell et al., 1997). T1L, rsT3D/T1LS1, T3D, and rsT3D produced \sim 1000-fold yields of viral progeny in L cells. However, only sialic acid-binding strains T3D and rsT3D were capable of efficiently infecting MEL cells, producing yields in each case of \sim 100-fold, whereas strains T1L and rsT3D/T1LS1 produced minimal yields of viral progeny in these cells ($<$ 10-fold). Together, these data indicate that recombinant reoviruses display replication characteristics indistinguishable from native strains.

Susceptibility of Attachment Protein σ 1 to Proteolytic Cleavage Attenuates Reovirus Intestinal Infection and Systemic Spread

The σ 1 protein exhibits strain-specific differences in susceptibility to cleavage following *in vitro* treatment with intestinal proteases to generate ISVPs (Nibert et al., 1995; Chappell et al., 1998). This difference in cleavage susceptibility segregates with a single amino acid polymorphism

in the tail domain of σ 1 (Figure 4A). Strains with a threonine at residue 249 in σ 1 are susceptible to cleavage by trypsin after Arg245, whereas those with an isoleucine at residue 249 are resistant to cleavage (Chappell et al., 1998). The importance of sequence polymorphism at residue 249 has been confirmed in studies using expressed protein (Chappell et al., 1998) and recoated core particles (Chandran et al., 2001) but not with intact virions.

To determine whether the single Thr-Ile polymorphism at residue 249 in σ 1 protein is sufficient to alter σ 1 cleavage susceptibility during treatment of virions with intestinal proteases to generate ISVPs, we used plasmid-based rescue to generate rsT3D- σ 1T249I, which differs from rsT3D by the presence of an isoleucine in σ 1 at residue 249 (Table S2). Purified virions of rsT3D and rsT3D- σ 1T249I were treated with trypsin and analyzed by SDS-PAGE. As expected, a digestion pattern consistent with formation of ISVPs (loss of σ 3 protein and generation of the δ fragment of μ 1C protein) was observed for both viruses (Figure 4B). However, the stability of rsT3D and rsT3D- σ 1T249I σ 1 proteins differed. The band corresponding to rsT3D σ 1 diminished in intensity immediately after trypsin addition until it was eventually undetectable (Figure 4B). However, the rsT3D- σ 1T249I σ 1 band was intact even after 60 min of digestion. Thus, the T249I polymorphism is an independent determinant of σ 1 cleavage susceptibility.

Proteolytic cleavage of σ 1 at a site adjacent to Thr249 releases the JAM-A-binding σ 1 head domain, leading to

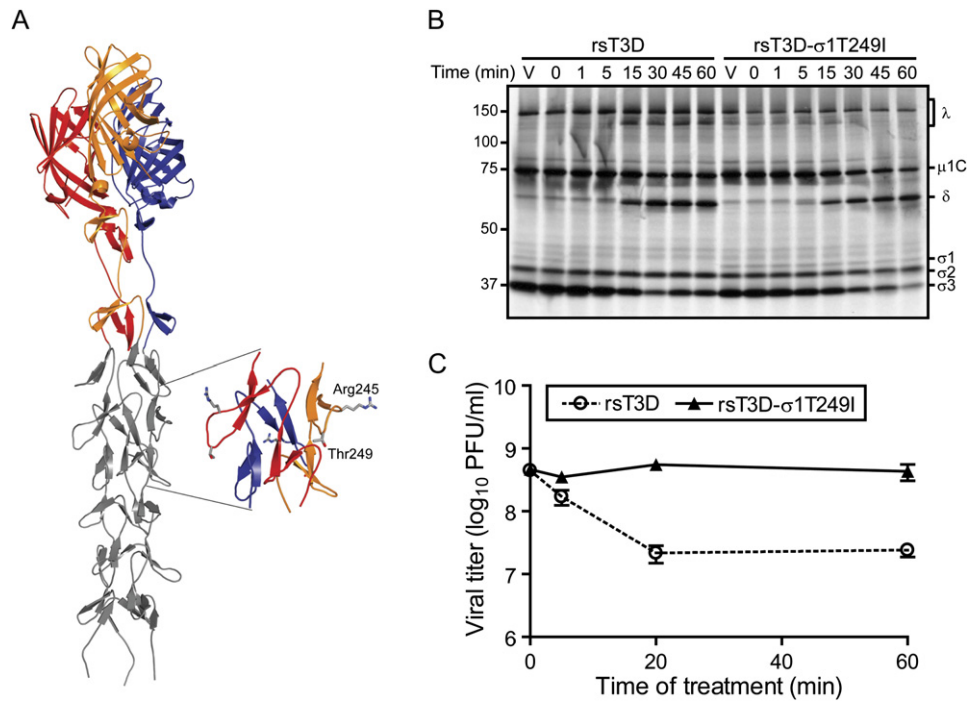


Figure 4. The $\sigma 1$ Protein of rsT3D- $\sigma 1$ T249I Is Resistant to Trypsin

(A) Model of $\sigma 1$ generated by adding five β spiral repeats to the N terminus of the crystallized fragment of $\sigma 1$ (Chappell et al., 2002). The three monomers in the crystallized fragment are shown in red, yellow, and blue; the model is shown in gray. The inset shows an enlarged view of the β spiral region in $\sigma 1$ that influences susceptibility of the molecule to cleavage by intestinal proteases (Chappell et al., 1998). Side chains of Arg245 and Thr249 are depicted in ball-and-stick representation.

(B) Electrophoretic analysis of viral structural proteins of rsT3D and rsT3D- $\sigma 1$ T249I during treatment with trypsin to generate ISVPs. Purified ³⁵S-methionine-labeled virions were treated with trypsin for the indicated intervals and loaded into wells of a 4%–20% polyacrylamide gradient gel. After electrophoresis, the gel was prepared for fluorography and exposed to film. Samples of untreated virions appear in the lanes labeled “V.” Viral proteins are labeled. Positions of molecular weight standards (in kDa) are indicated. The experiments shown are representative of two performed for each virus.

(C) Infectivity of rsT3D and rsT3D- $\sigma 1$ T249I during treatment with trypsin to generate ISVPs. Purified virions were treated with trypsin at 37°C for the intervals shown. Titers of virus in the treatment mixtures were determined by plaque assay. The results are presented as the mean viral titers for triplicate experiments. Error bars indicate SD.

diminished viral infectivity (Nibert et al., 1995). To test whether rsT3D and rsT3D- $\sigma 1$ T249I differ in infectivity after protease treatment to generate ISVPs, purified virions of each strain were exposed to trypsin for various intervals, and titers of infectious virus in the treatment mixtures were determined by plaque assay (Figure 4C). As observed with WT T3D in previous experiments (Nibert et al., 1995), rsT3D lost infectious titer rapidly after protease treatment. In contrast, titers of rsT3D- $\sigma 1$ T249I remained relatively stable throughout the assay time course. Loss of infectivity of rsT3D correlated with kinetics of $\sigma 1$ cleavage (compare Figures 4B and 4C), indicating that changes in viral infectivity after trypsin treatment are governed by the cleavage state of $\sigma 1$. Furthermore, both phenotypes are linked to a single $\sigma 1$ polymorphism at amino acid 249.

Reovirus strains T1L and T3D differ in the capacity to infect the murine intestine after peroral (PO) inoculation (Bodkin et al., 1989), a property that segregates with the viral S1 (encoding $\sigma 1$ and $\sigma 1s$) and L2 (encoding $\lambda 2$) genes (Bodkin and Fields, 1989). Exposure of T3D to an intestinal

wash results in $\sigma 1$ cleavage (Chappell et al., 1998), raising the possibility that failure of T3D to infect the intestine is in part attributable to $\sigma 1$ cleavage susceptibility. To test whether susceptibility of $\sigma 1$ to proteolytic cleavage is associated with diminished T3D growth in animals, we assessed the capacity of rsT3D and rsT3D- $\sigma 1$ T249I to infect the intestine and disseminate systemically following PO inoculation (Figure 5A). Newborn mice were inoculated perorally with each strain, and viral titers in the intestine and brain were determined at 4, 8, and 12 days after inoculation. At all time points tested, titers of rsT3D- $\sigma 1$ T249I in the intestine were greater than those produced by rsT3D. Furthermore, rsT3D- $\sigma 1$ T249I produced greater titers in the brain at days 8 and 12 than did rsT3D. However, when inoculated by the intracranial (IC) route, rsT3D and rsT3D- $\sigma 1$ T249I produced equivalent titers (Figure 5B), although rsT3D reached peak titers at earlier time points than did rsT3D- $\sigma 1$ T249I. These findings indicate that a Thr-Ile polymorphism at amino acid 249 in T3D $\sigma 1$ controls viral growth in the murine intestine and systemic spread to the CNS.

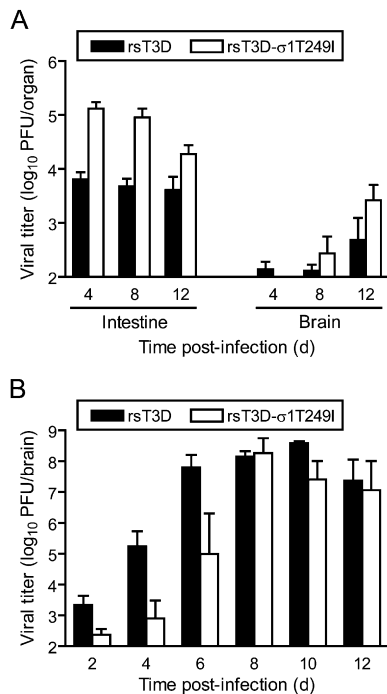


Figure 5. rsT3D-σ1T249I Infects the Murine Intestine and Disseminates to the CNS

Titers of rsT3D and rsT3D-σ1T249I after either PO (A) or IC (B) inoculation. Mice were inoculated with virus and euthanized at the indicated times postinoculation. Viral titers in organ homogenates were determined by plaque assay. The limit of detection was 100 PFU/ml of organ homogenate. Each data point represents the average viral titer from 3 to 12 mice. Error bars indicate SD.

Regulation of Reovirus Disassembly by a Single Polymorphism in Outer-Capsid Protein σ3

Previous studies identified a tyrosine-to-histidine substitution at amino acid 354 in T3D σ3 as a key regulator of the kinetics of virion-to-ISVP conversion *in vitro* (Wetzel et al., 1997) and viral resistance to growth inhibition by the cysteine protease inhibitor E64 (Baer and Dermody, 1997; Ebert et al., 2001). Tyr354 is located in the virion-distal lobe of σ3 adjacent to sites in the protein that are cleaved during formation of ISVPs (Ebert et al., 2002) (Figure 6A). The importance of this residue in viral replication has been deduced by analysis of reassortant viruses containing WT and mutant σ3 proteins (Wetzel et al., 1997; Ebert et al., 2001; Clark et al., 2006) and analysis of ISVPs recoated with WT and mutant forms of σ3 (Wilson et al., 2002; Clark et al., 2006).

To determine whether the Y354H mutation in σ3 is sufficient to confer enhanced virion-to-ISVP conversion and resistance to E64, we generated rsT3D-σ3Y354H (Table S2) and compared this virus to rsT3D for kinetics of σ3 proteolysis following protease treatment *in vitro*. Virions of each strain were treated with chymotrypsin for various intervals and processed for analysis of viral structural proteins by SDS-PAGE (Figure 6B). Treatment of rsT3D and rsT3D-σ3Y354H virions with chymotrypsin resulted in degradation of σ3 and cleavage of μ1C to form δ, indic-

ative of ISVP formation. Proteolysis of rsT3D-σ3Y354H σ3 during chymotrypsin treatment occurred with substantially faster kinetics than that of rsT3D σ3. This result indicates that amino acid 354 in σ3 protein is an independent determinant of virion susceptibility to proteolytic digestion and likely functions as an autonomous regulator of viral disassembly in cellular endosomes.

The role of σ3 mutation Y354H in virion disassembly *in cyto* was investigated by quantifying yields of rsT3D and rsT3D-σ3Y354H after 24 hr of growth in L cells treated with 0–200 μM E64 (Figure 6C). Both strains produced yields of ~1000 fold following growth in untreated cells. However, yields of rsT3D-σ3Y354H were ~100-fold greater than those of rsT3D following growth in cells treated with 200 μM E64 (the highest concentration tested). Therefore, a single mutation in σ3, Y354H, regulates resistance of reovirus to an inhibitor of cysteine proteases within cellular endosomes.

Transduction of GFP by a Recombinant Reovirus

To determine whether reoviruses capable of expressing a foreign gene can be recovered following plasmid transfection, we introduced sequences encoding GFP into the σ3 ORF of the T3D S4 plasmid (Figure 7A). In this configuration, GFP is expressed as a fusion protein incorporating amino acids 1–39 of σ3 protein at the N terminus. Recombinant virus rsT3D/S4-GFP was recovered following plasmid transfection of L cells stably expressing WT σ3 protein, which is required for propagation of this strain (Figure S1). RT-PCR analysis using primers specific for T3D S4 and GFP confirmed incorporation of a recombinant S4-GFP gene segment into rsT3D/S4-GFP (Figure 7B). Infection of L cells with rsT3D/S4-GFP resulted in expression of GFP and viral inclusion-forming proteins μNS and σNS but not σ3 (Figures 7C and 7D). The capacity of rsT3D/S4-GFP to express GFP was not altered through four passages (data not shown). These results demonstrate that reovirus can be engineered to express foreign genes.

DISCUSSION

The absence of DNA intermediates in the life cycle of RNA viruses poses a technical challenge to genetic analysis of viral phenotypes. Prior to the development of reverse genetics, or “marker rescue,” for RNA viruses of animals, in which plasmid-borne cDNAs of viral genomes initiate synthesis of replication-competent RNAs, classical Darwinian methods were used to select viral mutants that could be subjected to correlative genetic studies—so-called “forward genetics.” However, reverse genetics technology permits testing of tightly focused, rational hypotheses about complex questions of virus structure, virus-cell interactions, and viral pathogenesis through direct engineering of the viral genome without a need to devise complicated selection strategies for isolation of viral mutants. Furthermore, reverse genetics of RNA viruses has supported rapid generation of vaccines against these and other infectious agents and propelled their use as gene

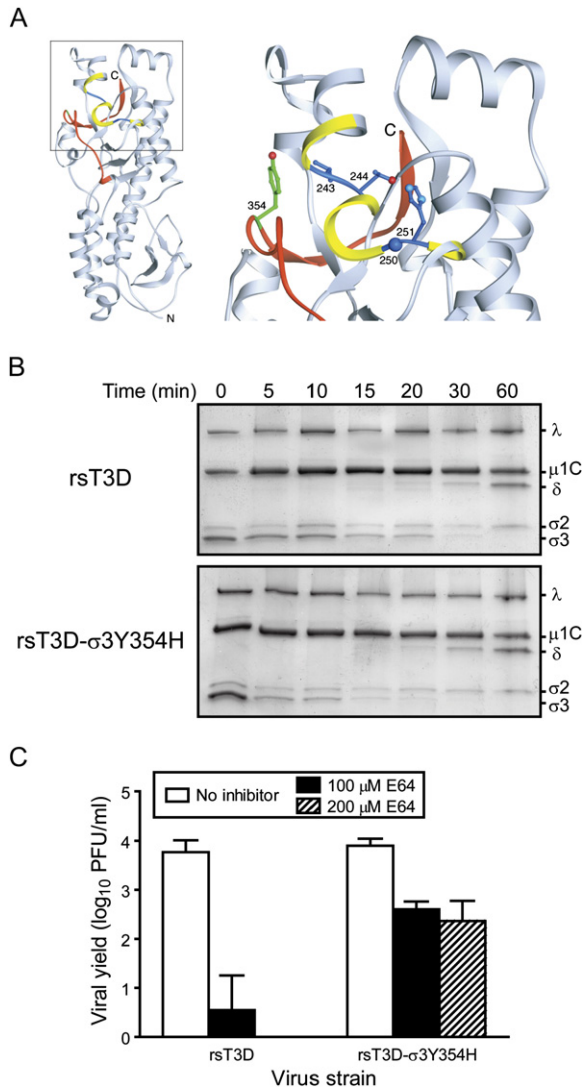


Figure 6. A Single Mutation in Outer-Capsid Protein σ_3 Accelerates Proteolytic Disassembly of Reovirus

(A) Crystal structure of T3D σ_3 (Olland et al., 2001), in which cathepsin L cleavage sites are depicted in blue between amino acids 243 and 244 and between 250 and 251 (Ebert et al., 2002). Surrounding residues, from amino acids 241 to 253, are shown in yellow. The C-terminal residues of σ_3 , from amino acids 340–365, are colored red. Tyr354, which is altered in several PI (Wetzels et al., 1997), D-EA (Ebert et al., 2001), and ACA-D viruses (Clark et al., 2006), is shown in green. The virion-distal end of σ_3 is at the top of the figure, and the virion-proximal end and N terminus are at the bottom. The inset shows an enlarged view of the boxed region of σ_3 using the same color scheme. Side chains of amino acids 243, 244, 250, 251, and 354 are depicted in ball-and-stick representation.

(B) Chymotrypsin treatment of rsT3D and rsT3D- σ_3 Y354H. Purified virions were treated with chymotrypsin for the indicated intervals and loaded into wells of 10% polyacrylamide gels. After electrophoresis, the gels were stained with Coomassie blue. Viral proteins are labeled. The experiments shown are representative of two performed for each virus.

(C) Growth of rsT3D and rsT3D- σ_3 Y354H in L cells treated with E64. L cells were preincubated in medium with or without E64 at the concentrations shown. The medium was removed, cells were adsorbed with

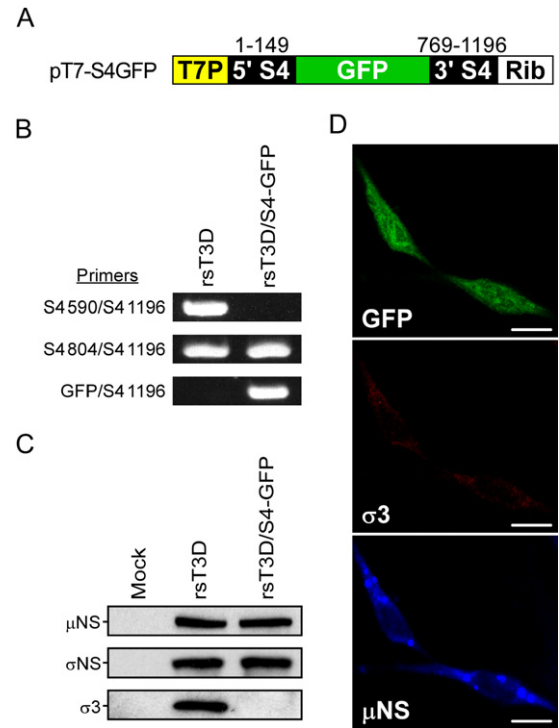


Figure 7. Expression of GFP by rsT3D/S4-GFP

(A) Schematic of pT7-S4GFP. The GFP ORF is flanked by S4 gene nucleotides 1–149 and 769–1196.

(B) RT-PCR analysis of rsT3D and rsT3D/S4-GFP. Viral dsRNA was extracted from purified virions and subjected to RT-PCR using primers specific for T3D S4 and GFP sequences. Numbers delineate the S4 RNA nucleotide position corresponding to the 5' end of S4-specific primers.

(C) Viral protein expression in cells infected with rsT3D/S4-GFP. L cells were infected with rsT3D or rsT3D/S4-GFP at an MOI of 1 PFU per cell and incubated for 24 hr. Cell lysates were analyzed by immunoblotting using antibodies specific for μ NS, σ NS, and σ_3 proteins.

(D) Image analysis of cells infected with rsT3D/S4-GFP. L cells were infected with rsT3D/S4-GFP, stained with antibodies specific for μ NS (blue) and σ_3 (red) proteins at 24 hr postinfection, and imaged by confocal laser scanning microscopy. Scale bar, 10 μ M.

delivery vehicles (Blaney et al., 2006; Horimoto and Kawaoka, 2006; Riezebos-Brilman et al., 2006).

We developed a fully plasmid-based reverse genetics technology for mammalian reoviruses. This system permits selective introduction of desired mutations into cloned cDNAs encoding each of the ten viral gene segments, followed by isolation of mutant viruses from cells transfected with the plasmid constructs. Moreover, gene segment cDNAs can be manipulated to facilitate expression of a transgene. Importantly, recombinant viruses are generated without a requirement for helper virus and free of any selection. Thus, this new technology provides

virus for 1 hr, and fresh medium with or without E64 was added. After 24 hr incubation, viral titers in cell lysates were determined by plaque assay. The results are presented as the mean viral yields, calculated by dividing titer at 24 hr by titer at 0 hr for each concentration of E64, for triplicate experiments. Error bars indicate SD.

a means to directly and precisely engineer the viral genome in the context of infectious virus.

We used the newly developed plasmid-based reovirus reverse genetics system to engineer mutations in the $\sigma 1$ and $\sigma 3$ proteins. These proteins form part of the viral outer capsid, which is responsible for numerous major events in reovirus interaction with the cell and host, including attachment, disassembly within endosomes, penetration of cell membranes, induction of apoptosis, growth in the intestine, pathways of spread, neurovirulence, and tropism within the CNS (for reviews, see Chandran and Nibert, 2003; O'Donnell et al., 2003; Guglielmi et al., 2006). Therefore, we initially applied reverse genetics technology to the study of outer-capsid proteins to better understand how these proteins mediate critical steps in reovirus replication and disease.

The infectivity of ISVPs of reovirus strain T1L in L cells is approximately 10-fold greater than that of T3D ISVPs (Nibert et al., 1995). This difference in infectivity is hypothesized to be a direct result of $\sigma 1$ cleavage (Nibert et al., 1995; Chappell et al., 1998), presumably due to removal of the JAM-A-binding region of the molecule. Although the T249I substitution in expressed T3D $\sigma 1$ renders it resistant to cleavage by trypsin (Chappell et al., 1998), until now it has not been possible to define the role of $\sigma 1$ cleavage in T3D infectivity for lack of means to generate a mutant T3D virus with the T249I change. This virus has been generated using reverse genetics, and our findings indicate that cleavage susceptibility of viral attachment protein $\sigma 1$ due to a single polymorphism at amino acid position 249 is the basis for reduced infectivity of T3D ISVPs relative to virions (Figure 4C) and contributes to diminished growth of T3D in the murine intestine following PO inoculation (Figure 5A).

Previous studies of T3D-derived reovirus strains with altered disassembly kinetics point to a critical role for sequences in the virion-distal, C-terminal lobe of $\sigma 3$ in susceptibility to acid-dependent proteolysis. A C-terminal Y354H mutation in $\sigma 3$ protein of strain T3D was selected during persistent infection of L cells (PI viruses) (Wetzel et al., 1997) and by serial passage of virus in L cells treated with either E64 (D-EA viruses) (Ebert et al., 2001) or ammonium chloride (ACA-D viruses) (Clark et al., 2006). Using reovirus reverse genetics, the Y354H substitution was introduced into a WT T3D genetic background, and the resultant virus, rsT3D- $\sigma 3$ Y354H, demonstrated accelerated kinetics of $\sigma 3$ cleavage (Figure 6B) and diminished sensitivity to the growth-inhibitory effects of E64 (Figure 6C). Residue 354 is located in a position thought to be important for controlling access to protease-hypersensitive regions in $\sigma 3$, residues 208–214 and 238–242, thereby influencing $\sigma 3$ cleavage kinetics (Jané-Valbuena et al., 2002). Therefore, it appears that residue 354 in $\sigma 3$ is a gatekeeper for the viral outer capsid, serving to regulate the balance between viral stability and an irreversible, proteolytically triggered disassembly cascade committing the virion particle to either replication or inactivation.

We exploited the reovirus reverse genetics system to develop a gene-delivery vehicle by replacing the $\sigma 3$ ORF in

the S4 plasmid with a GFP-encoding cDNA (Figure 7). The resultant virus, rsT3D/S4-GFP, expresses GFP through successive passages in cell culture. These results reveal the potential for exploitation of reovirus as a gene-transduction vector with application in the development of new mucosal vaccines, more effective oncolytic agents (Coffey et al., 1998), and high-level expression of foreign genes in animal cells. Ideal reovirus vectors will contain stable $\sigma 1$ proteins and combine excellent extracellular stability with highly efficient intracellular disassembly. We find that each of these parameters can be independently adjusted through strategic alterations in outer-capsid proteins. Manipulation of inner-capsid proteins and the genomic RNA itself should allow construction of viruses able to circumvent other aspects of virus-cell and virus-host interactions that pose potential barriers to antigen and gene delivery by reovirus.

Our results indicate that productive viral infection is established in a small fraction of L cells, approximately 1 in 10^5 – 10^6 cells, transfected with plasmids encoding the ten reovirus gene segments (Figure 1D). Thus, the reovirus plasmid-based marker rescue system is suited to the isolation of viable viral clones by plaque assay, followed by expansion in cell culture to attain quantities of virus sufficient for phenotypic analyses. Manipulations that severely cripple viral replication or particle assembly are more challenging to study because these changes may prohibit virus isolation. However, recovery of a GFP-expressing virus, rsT3D/S4-GFP, demonstrates that marker rescue of lethal mutations is possible by transcomplementation (in this case with WT $\sigma 3$ protein) (Figure 7 and Figure S1). This result also agrees with our previous finding that inhibition of reovirus replication by RNAi-mediated reduction of viral protein synthesis is reversible by transcomplementation with ectopically expressed WT protein (Kobayashi et al., 2006). Furthermore, transcomplementation allows precise definition through systematic mutational analysis of functional domains in reovirus proteins that are essential for viral replication (Kobayashi et al., 2006). It should be possible to apply this technique to the new marker rescue system for delineation of structure-function relationships in reovirus proteins and RNA.

Quantitative success of plasmid-initiated reovirus infection in this reverse genetics system probably is not limited by the amount of template or transfection efficiency, since the molar ratio of plasmid to target cell is approximately 5×10^4 , and increasing the amount of plasmid does not effect higher viral yields from cotransfection lysates (data not shown). Furthermore, it does not appear that infection efficiency is limited by the absence of viral replication proteins during early steps of replication because high-level expression of the replication proteins $\mu 2$, μNS , and σNS , which collaborate to form viral inclusions in infected cells (Mbisa et al., 2000; Broering et al., 2002; Becker et al., 2003; Miller et al., 2003), did not enhance viral recovery (data not shown). Perhaps the presence of other viral or cellular factors associated with natural infection by intact virion particles is required for maximal reovirus infectivity.

Presently, no entirely plasmid-based reverse genetics system has been described for any other dsRNA virus of animals. Although each genus within this constellation of viruses bears unique biologic characteristics and physiochemical properties, there are nonetheless numerous unifying features of virion particle structure and replication mechanisms that should allow principles and techniques established in this study to be applied broadly across the group. We expect that new insights into mammalian reovirus replication learned with the use of this reverse genetics system will be quickly extrapolated to other *Reoviridae* family members, leading to accelerated development of analogous marker-rescue technologies for those viruses.

EXPERIMENTAL PROCEDURES

Cells and Viruses

L cells and HeLa cells were maintained as described (Barton et al., 2001a). Reovirus strains T1L and T3D are laboratory stocks originally obtained from Dr. Bernard Fields. Virus was purified after growth in L cells by CsCl-gradient centrifugation (Furlong et al., 1988). Purified ³⁵S-methionine-labeled virions were prepared as described (Nibert et al., 1995). Attenuated vaccinia virus strain rDIs-T7pol expressing T7 RNA polymerase was propagated in chick embryo fibroblasts as described (Ishii et al., 2002).

Plasmid Construction

Full-length reovirus cDNAs were amplified by RT-PCR using viral dsRNA extracted from purified virions as template. Amplified cDNAs were initially cloned into pBluescript II SK (-) (Stratagene) for the T3D L1, L2, and L3 genes or pCR 2.1 (Invitrogen) for the T3D M1, M2, M3, S1, S2, S3, and S4 genes and the T1L S1 gene (Table S3). To generate pT7-L1T3D, pT7-L2T3D, pT7-L3T3D, pT7-M1T3D, pT7-M2T3D, pT7-M3T3D, pT7-S2T3D, pT7-S3T3D, and pT7-S4T3D, viral cDNA-containing fragments were subcloned into p3E5EGFP (Watanabe et al., 2004). Viral cDNAs fused at their native 5' termini to the phage T7 RNA polymerase promoter were inserted into p3E5EGFP by partial or complete replacement of plasmid sequences encoding GFP and the Ebola virus leader and trailer, resulting in ligation of native 3' termini to the HDV ribozyme sequence. To generate pBacT7-S1T3D and pBacT7-S1T1L, encoding the T3D S1 and T1L S1 genes, respectively, S1 cDNAs fused to the T7 promoter and a portion of the HDV ribozyme were first cloned into the BseRI site of p3E5EGFP, and fragments containing the S1 gene flanked 5' by the T7 promoter and 3' by the HDV ribozyme and T7 terminator sequences were subcloned into the XbaI site of pBacPAK8 (Clontech). pBacT7-S1T3D and pT7-S4T3D were used as templates to generate mutant constructs pBacT7-S1T3DT249I and pT7-S4T3DY354H, respectively, by introduction of specific nucleotide substitutions using the QuickChange site-directed mutagenesis kit (Stratagene) (Table S4). To generate pT7-S4GFP, S4 nucleotide sequences 150–768 within pT7-S4T3D were replaced with the GFP ORF. Nucleotide sequences of recombinant plasmids were confirmed by DNA sequencing. Detailed description of cloning strategies is provided in the Supplemental Data.

Plasmid Transfection and Recovery of Recombinant Virus

Monolayers of L cells at approximately 90% confluence (3×10^6 cells) in 60 mm dishes (Costar) were infected with rDIs-T7pol at an MOI of ~ 0.5 TCID₅₀. At 1 hr postinfection, cells were cotransfected with ten plasmid constructs representing the cloned T3D genome—pT7-L1T3D (2 μ g), pT7-L2T3D (2 μ g), pT7-L3T3D (2 μ g), pT7-M1T3D (1.75 μ g), pT7-M2T3D (1.75 μ g), pT7-M3T3D (1.75 μ g), pBacT7-S1T3D (2 μ g), pT7-S2T3D (1.5 μ g), pT7-S3T3D (1.5 μ g), and pT7-S4T3D (1.5 μ g)—using 3 μ l of TransIT-LT1 transfection reagent (Mirus) per microgram of plasmid DNA. Following 0–5 days of incubation, recombinant virus was isolated from transfected cells by plaque puri-

fication on monolayers of L cells (Virgin et al., 1988). Electrophoretic analysis of viral dsRNA was performed as described (Wilson et al., 1996). Confirmation of mutations in the S1, S4, and L1 genes of recombinant viruses was performed using the Onestep RT-PCR kit (Qiagen), gene-specific primer sets, and viral dsRNA extracted from purified virions as template. PCR products were analyzed following electrophoresis in Tris-borate-EDTA agarose gels or purified and subjected directly to sequence analysis.

Infectious Center and Viral Yield Assays

L cells were cotransfected with ten plasmids corresponding to the T3D genome as described. For infectious center assays, transfected cells were released from plates by trypsin treatment at various intervals posttransfection, washed, counted, diluted, and applied directly to monolayers of untreated L cells (Dermody et al., 1995), which were processed for plaque assay (Virgin et al., 1988). For viral yield assays, transfected L cells were lysed by freezing and thawing, and viral titers in cell lysates were determined by plaque assay (Virgin et al., 1988).

Immunofluorescence Detection of Reovirus Infection

Parental L cells or L cell transfectants selected for stable expression of $\sigma 3$ protein (5×10^4) were plated on glass coverslips in 24-well plates (Costar) and infected at an MOI of 10,000 (T3D and rsT3D) or 20,000 (rsT3D/S4-GFP) particles per cell. Following incubation at 37°C for various intervals, cells were fixed and stained for μ NS and $\sigma 3$ proteins as described (Maginnis et al., 2006). Images were acquired using a Zeiss LSM 510 META inverted confocal system (Carl Zeiss) with a Zeiss inverted Axiovert 200M microscope and a Plan-APOCHROMAT 63 \times /1.4 NA oil immersion DIC objective. Images were processed using MetaMorph image analysis software (Molecular Devices).

Infectivity of Recombinant Viruses

Monolayers of L or L- $\sigma 3$ cells (2.5 to 5×10^5) in 24-well plates or suspension cultures of MEL cells (5×10^5 cells/ml) were infected with virus at an MOI of 1–2 PFU/cell. After 1 hr adsorption at room temperature, the inoculum was removed, cells were washed twice with PBS, and fresh medium was added. Cells were incubated at 37°C for various intervals, and viral titers in cell lysates were determined by plaque assay (Virgin et al., 1988).

Analysis of Viral Capsid Proteins after Protease Treatment

Purified virions at a concentration of either 2×10^{12} particles/ml (trypsin) or 9×10^{12} particles/ml (chymotrypsin) were digested with either 50 μ g/ml of N α -p-tosyl-L-sulfonyl phenylalanyl chloromethyl ketone-treated bovine trypsin (Sigma) or 200 μ g/ml of N α -p-tosyl-L-lysine chloromethyl ketone-treated bovine α -chymotrypsin (Sigma) for various intervals at either 25°C (trypsin) or 8°C (chymotrypsin) as described (Nibert et al., 1995; Wetzel et al., 1997). Protease digestion was stopped by adding either 0.5 mg/ml soybean trypsin inhibitor (trypsin) (Sigma) or 5 mM phenylmethyl-sulfonyl fluoride (chymotrypsin) (Sigma) to the treatment mixtures and cooling at 0°C. Viral proteins were resolved by SDS-PAGE and visualized by either autoradiography (Nibert et al., 1995) or staining with Coomassie blue (Wetzel et al., 1997).

Infection of Mice

Newborn C57/BL6 mice weighing 2.0–2.5 grams (2–4 days old) were inoculated perorally or intracranially with 10^3 or 10^2 PFU, respectively, of purified reovirus virions diluted in PBS. PO inoculations (50 μ l) were delivered intragastrically as described (Rubin and Fields, 1980). IC inoculations (5 μ l) were delivered to the left cerebral hemisphere using a Hamilton syringe and 30-gauge needle (Tyler et al., 1985). At various intervals following inoculation, mice were euthanized, and organs were harvested into 1 ml of PBS and homogenized by freezing, thawing, and sonication. Viral titers in organ homogenates were determined by plaque assay (Virgin et al., 1988). Animal husbandry and experimental procedures were performed in accordance with Public Health Service policy and approved by the Vanderbilt University School of Medicine Institutional Animal Care and Use Committee.

Growth of Virus in Cells Treated with E64

Monolayers of L cells (2×10^5) in 24-well plates were preincubated in medium supplemented with 0–200 μ M E64 (Sigma) for 4 hr. The medium was removed, and cells were adsorbed with virus at an MOI of 2 PFU/cell. After incubation at 4°C for 1 hr, the inoculum was removed, cells were washed with PBS, and 1 ml of fresh medium supplemented with 0 to 200 μ M E64 was added. Cells were incubated at 37°C for 24 hr and frozen and thawed twice. Viral titers in cell lysates were determined by plaque assay (Virgin et al., 1988).

Generation of σ 3-Expressing Cells

L cells stably expressing σ 3 protein (L- σ 3 cells) were selected by transfection of cells with pCXN-S4T3D, which encodes the entire T3D σ 3 ORF, and incubation in the presence of 1 mg/ml of geneticin (Invitrogen).

Supplemental Data

The Supplemental Data include Supplemental Experimental Procedures, four supplemental tables, and one supplemental figure and can be found with this article online at <http://www.cellhostandmicrobe.com/cgi/content/full/1/2/147/DC1/>.

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Accession Numbers

GenBank accession numbers for cDNAs corresponding to the T3D L1, L2, L3, M1, M2, M3, S1, S2, S3, and S4 genes and the T1L S1 gene are provided in [Table S3](#).

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