Mammalian reoviruses are nonenveloped viruses with a long, filamentous attachment protein that dictates disease phenotypes following infection of newborn mice and is a structural homologue of the adeno virus attachment protein. Reoviruses use junctional molecule 1 (JAM1) as a serotype-independent cellular receptor. JAM1 is a broadly expressed immunoglobulin superfamily protein that forms stable homodimers and regulates tight-junction permeability and lymphocyte trafficking. We employed a series of structure-guided binding and infection experiments to define residues in human JAM1 (hJAM1) important for reovirus-receptor interactions and to gain insight into mechanisms of reovirus attachment. Binding and infection experiments using chimeric and domain deletion mutant receptor molecules indicate that the amino-terminal D1 domain of hJAM1 is required for reovirus attachment, infection, and replication. Reovirus binding to hJAM1 occurs more rapidly than homotypic hJAM1 association and is competed by excess hJAM1 in vitro and on cells. Cross-linking hJAM1 diminishes the capacity of reovirus to bind hJAM1 in vitro and on cells and negates the competitive effects of soluble hJAM1 on reovirus attachment. Finally, mutagenesis studies demonstrate that residues intimately associated with the hJAM1 dimer interface are critical for reovirus interactions with hJAM1. These results suggest that reovirus attachment disrupts hJAM1 dimers and highlight similarities between the attachment strategies of reovirus and adenovirus.

Mammalian reoviruses are prototype members of the Reoviridae family of viruses. They are nonenveloped viruses that contain a genome of 10 double-stranded RNA segments (1). Reoviruses have been isolated from many mammalian species, including humans; however, severe disease is rare and usually restricted to the very young (2). Neonatal mice are exquisitely susceptible to reovirus infection and have been employed for studies of viral pathogenesis with particular emphasis on central nervous system tropism and corollary disease phenotypes. Following oral inoculation of newborn mice, serotype 1 (T1) reovirus strains spread hematogenously from the intestine to the central nervous system and demonstrate tropism for epidermal cells (3–5). In contrast, serotype 3 (T3) reovirus spreads via neural routes to the central nervous system, where they infect neurons (3–6). As a result of these differences in cell tropism, T1 strains cause nonlethal hydrocephalus, whereas T3 strains cause lethal encephalitis. Reassortant genetics defined the viral S1 gene segment as the primary genetic correlate of these tropism and disease phenotypes (3–5).

The S1 gene segment encodes the reovirus attachment protein, σ1 (7, 8). The trimeric σ1 protein exhibits head-and-tail morphology (9–13) and inserts into the virion capsid at the icosahedral 5-fold symmetry axes. The adeno virus attachment protein, fiber, exhibits similar morphology and virion insertion (14). A major portion of the σ1 tail, including residues important for carbohydrate binding (15), folds into a triple β-spiral structural motif (13). This motif consists of repeating units of short β-strands that previously had only been seen in the “shaft” domain of adeno virus fiber (16). Similarities also exist between the “knob” domain of fiber and the σ1 head, which both form globular structures composed of unique eight-stranded β-barrels (13). Evidence gathered from biochemical and genetic studies previously defined the σ1 head as the viral determinant of proteinaceous cellular receptor engagement (3–5, 17). The strong association of the S1 gene segment with serotype-dependent differences in reovirus tropism and disease led to the hypothesis that reovirus serotypes usurp distinct cell surface molecules for attachment and infection.

To better understand the contributions of cellular receptors in reovirus pathogenesis, we used an expression-cloning approach to identify junctional adhesion molecule 1 (JAM1) as a receptor for reovirus and demonstrated that reovirus directly engages JAM1 in a bimolecular interaction via the head domain of σ1 (17). JAM1 is a receptor for both T1 and T3 reovirus strains in culture (17); however, the in vivo roles of JAM1 in reovirus tropism and pathogenesis have not been defined.

The abbreviations used are: JAM1, junctional adhesion molecule 1; mJAM1, mouse JAM1; hJAM1, human JAM1; hCAR, human coxsackievirus and adenovirus receptor; CHO, Chinese hamster ovary; T1L, type 1 Lang; Ad 5-GFP, green fluorescent protein-encoding serotype 5 adenovirus; mAb, monoclonal antibody; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; B82, bis(sulfsuccinimidyl) suberate; ELISA, enzyme-linked immunosorbent assay.
JAM1 is a type 1 transmembrane protein consisting of two extracellular Ig-like domains, termed D1 and D2, a single transmembrane segment, and a short cytoplasmic tail (18, 19). JAM1 is expressed in a variety of tissues, including epithelial and endothelial barriers (18–20), where it is thought to regulate tight junction permeability and mediate lymphocyte trafficking (18–21). The crystal structures of murine and human homologues of JAM1 (mJAM1 and hJAM1, respectively), both of which are functional reovirus receptors (17), indicate that JAM1 forms homodimers via extensive hydrophobic and ionic contacts between apposing D1 domains (22, 23). Residues that facilitate interdimer interactions are strictly conserved between mJAM1 and hJAM1 (22, 23). JAM1-D1 dimers are highly stable and thought to be physiologically relevant, perhaps functioning in tight junction barrier integrity or diapedesis of inflammatory cells (22–24).

The contacts that facilitate JAM1 dimerization are interesting in that they occur via the GFCC’ face of the D1 Ig-like domain (22, 23). The only other molecules demonstrated to form homodimers using similar interdimer contacts are the human coxsackievirus and adenovirus receptor (hCAR) and CD2 (25–27). The structure of hCAR in complex with the adenovirus fiber knob revealed that fiber engages the D1 domain of hCAR using residues involved in hCAR homodimer formation and that knob mimics the hCAR-hCAR interaction (16, 25). Biophysical evidence suggests that fiber-hCAR interactions are thermodynamically favored over hCAR-hCAR interactions, providing support for a model in which residues in the hCAR dimer interface preferentially bind fiber over hCAR (28). The structural similarities between the reovirus and adenovirus attachment proteins and between their cognate receptors, paired with the absolute conservation of residues in mJAM1 and hJAM1 that mediate homodimer formation, suggest that reovirus engages the D1 domain of hJAM1 and that residues involved in hJAM1 dimerization are important for reovirus entry (23). This hypothesis was formally tested in the current study.

For these experiments, we generated chimeric receptor molecules consisting of reciprocal domain exchanges between hCAR and hJAM1 and single domain hJAM1 deletion mutants and tested the capacity of these constructs to support reovirus binding and infection. We performed complementary in vitro and cellular competition binding studies and hJAM1 dimer cross-linking experiments to assess the effects on reovirus attachment. Finally, we generated a series of hJAM1 point mutants to define specific residues important for reovirus-hJAM1 interactions. The results of these structure-guided approaches reveal that residues in the hJAM1 D1 domain within and proximal to the dimer interface are critical for reovirus-hJAM1 interactions. These findings more clearly define the molecular basis of reovirus binding to hJAM1 and highlight potential mechanisms of reovirus attachment. Moreover, they provide biological and biophysical evidence that reovirus and adenovirus use remarkably similar attachment strategies.

**EXPERIMENTAL PROCEDURES**

**Cells, Viruses, and Antibodies—** Spinner adapted L929 (L) cells were maintained in Dulbecco’s modified Eagle’s medium supplemented to contain 5% fetal bovine serum, 2 mM t-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Reovirus strain type 1 Lang (T1L) is a laboratory stock. Strain T12A– is a non-sialic acid-binding monoreassortant virus (29). Reoviruses were purified from infected L cells as previously described (9). Particle concentrations were determined by spectrophotometry at 260 nm using a conversion factor of 2.1 × 10¹⁴ particles/mlA₂₆₀. Particle/plaque-forming unit ratios for T1L were 100:1. Particle/fluorescent focus unit ratios for T1L were 10,000:1. Green fluorescent protein-encoding serotype 5 adenovirus (Ad5-GFP) was provided by Dr. Jeffrey Bergelson (University of Pennsylvania). hJAM1-specific monoclonal antibody (mAb) J10.4 and rabbit polyclonal hJAM1 antiserum were provided by Dr. Charles Parkos (Emory University).

**Generation of Chimeric and Mutant Receptor Constructs—** Nomenclature of chimeric and deletion mutant constructs indicates exchanged or deleted domains relative to wild-type hCAR or hJAM1 from amino to carboxy termini. Chimeric receptor CJJ (hCAR residues 1–141; hJAM1 residues 133–299) and deletion mutants JAJ (hJAM1 residues 133–234 deleted) and AJJ (hJAM1 residues 235–299 deleted) were generated using PCR to insert a HindIII endonuclease restriction site at the 3′ or 5′ end of respective amino-terminal or carboxyl-terminal receptor fragments. Chimeras CJJ (hJAM1 residues 1–128, hCAR residues 138–237, hJAM1 residues 235–299) and full-length D1 point mutant receptors were generated by overlap extension PCR. All chimeric and mutant receptor PCR products were digested with restriction endonucleases and ligated into plasmid pcDNA3.1+ (Invitrogen). The fidelity of cloning was confirmed by automated sequencing. The PCR primers used to generate chimeric and deletion mutant receptors are shown in Table I. The PCR primers used for point mutant coding changes are shown in Table II.

**Transient Transfection and Infection of CHO Cells—** CHO cells were transiently transfected with empty vector or plasmids encoding wild-type, chimeric, or deletion mutant receptors using LipofectAMINE and PLUS reagent (Invitrogen) as previously described (23). Cells were incubated for 24 h to allow receptor expression and then infected with reovirus T1L at multiplicities of infection of 1 fluorescent focus unit/cell and 1 plaque-forming unit/cell for fluorescent focus and plaque assays, respectively. For fluorescent focus assays, infected cells were processed for indirect immunofluorescence as previously described (29). Images were captured at ×20 magnification using a Leica DM IRB inverted microscope. For plaque assays, viral titers in cell lysates were determined at 0 and 24 h after adsorption as previously described (30).

**Flow Cytometric Analysis of Receptor Expression and Virus Binding—** CHO cells were transiently transfected and incubated for 24 h to allow receptor expression. Cells were detached from plates by incubation with 20 mM EDTA in PBS. Cells (1 × 10⁶) were incubated with hCAR- or hJAM1-specific antisera at dilutions of 1:750 or 1:1000, respectively, or incubated with reovirus T1L or T3SA– (1 × 10⁵ particles/cell) on ice for ~60 min. Virus-adsorbed cells were washed with PBS and incubated with a combined T1LT3D antiserum (21) at 1:1000 dilution on ice for ~60 min. All samples were washed with PBS and incubated with phycoerythrin-conjugated goat anti-rabbit IgG secondary antisera (Molecular Probes, Inc.) at a 1:1000 dilution on ice for ~30 min. Cells were washed twice with PBS and fixed with 2% paraformaldehyde in PBS. Cells were analyzed using a FACSscan flow cytometer (Becton-Dickinson).

**Expression and Purification of Soluble Receptor Constructs—** Soluble ectodomains of wild-type and point mutant hJAM1 constructs were fused to an amino-terminal glutathione S-transferase affinity tag via a thrombin cleavage site and purified as described (23). Nucleotide sequences corresponding to residues 27–233 of wild-type hJAM1 and

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**FIG. 1.** Chimeric and deletion mutant receptor constructs for studies of reovirus binding and growth. Ig superfamilies proteins hCAR (red) and hJAM1 (blue) were used to generate chimeric receptor constructs in which Ig-like domains were reciprocally exchanged. Single Ig-like domains of hJAM1 also were deleted. Nomenclature indicates origin or deletion of domains D1, D2, and cytoplasmic tail (left to right) relative to wild-type hCAR or hJAM1.
hJAM1 point mutants were cloned by PCR, digested with restriction endonucleases, and ligated into pGEX-4T-3 (Amersham Biosciences) for bacterial transformation. Bacteria were cultured in Luria-Bertani broth at 37 °C with shaking, and protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactoside (Amersham Biosciences). Bacteria were harvested by centrifugation and lysed by sonication in the presence of protease inhibitor mixture (Roche Molecular Biochemicals). Glutathione S-transferase-hJAM1 constructs were purified from bacterial lysates by glutathione affinity chromatography. Soluble wild-type and point mutant hJAM1 ectodomains were liberated from the glutathione resin by thrombin cleavage (20 units/ml) at room temperature overnight.

In Vitro hJAM1 and Reovirus Binding Analysis—hJAM1 (500 µg/ml) and reovirus TIL (1.1 × 10^{11} particles/ml) in PBS were biotinylated by incubation in 200 µg/ml EZ-Link Sulfo-NHS-LC-biotin (Pierce) at room temperature for 60 min. Unincorporated biotin was removed by exhaustive dialysis against PBS. ELISA plates were coated with 20 µl solutions of soluble hJAM1, cross-linked hJAM1, point mutant hJAM1, or bovine serum albumin (2 µg/ml) in pH 9.6 carbonate-bicarbonate buffer (Sigma). Plates were blocked by incubation with 2% (w/v) bovine serum albumin, 0.05% Triton X-100, PBS. For kinetic analyses, biotinylated reovirus (2.5 × 10^{11} particles/ml) in pH 9.6 carbonate-bicarbonate buffer was incubated with hJAM1-coated plates for 0, 5, 15, 30, 60, 120, 180, 240, or 300 min, and biotinylated reovirus (2.5 × 10^{10}, 5.0 × 10^{10}, 1.0 × 10^{11}, or 2 × 10^{11} particles/ml or 0.042, 0.083, 0.16, or 0.33 nm, respectively) in blocking buffer was incubated with hJAM1-coated plates for 0, 5, 15, 30, 60, 120, 180, and 240 min at 37 °C. As controls for specificity, binding of 80 nM biotinylated hJAM1 in the presence of a 100-fold excess of unlabeled hJAM1 at 180 min and binding of 0.33 nM reovirus (2.5 × 10^{11} particles/ml) in the presence of 20 µg/ml hJAM1-specific mAb J10.4 or hCAR-specific mAb RmcB (32) were assessed. For competition and equilibrium analyses, biotinylated reovirus (2 × 10^{11} particles/ml; 0.33 nm) in blocking buffer was incubated with hJAM1-, cross-linked hJAM1-, or point mutant hJAM1-coated plates in the presence or absence of excess soluble hJAM1 or cross-linked hJAM1 at 37 °C for 180 min. Binding in the presence of 20 µg/ml hJAM1-specific mAb J10.4 was tested as a control for specificity. All plates were washed twice with 0.05% Triton X-100/PBS and once with PBS and incubated with 2 µg/ml horseradish peroxidase (HRP)-conjugated streptavidin (Amersham Biosciences) in blocking buffer at room temperature for 30 min. Plate-bound biotinylated hJAM1 or reovirus was detected following incubation with HRP substrate (ABTS (2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid), Sigma) and analysis by spectrophotometry at 405 nm. For all conditions tested, no binding to bovine serum albumin-coated plates was detected.

**Virus Radioligand Binding Assays**—Radioligand binding assays were performed as previously described (29). Reovirus TIL was metabolically labeled with Easy Tag TM EXPRE35S35S (PerkinElmer Life Sciences) and purified as described (9). HeLa cells were detached from plates by incubation with 20 mM EDTA, resuspended in Dulbecco’s PBS (Invitrogen) supplemented with metabolic inhibitors (10 mM NaN₃, 5 mM 2-deoxyglucose, and 2 mM NaF), and incubated at 37 °C for 30 min to deplete cellular ATP and block receptor-mediated endocytosis (29, 33). Cells (1 × 10^9) were incubated with 35S-labeled reovirus T1L (1 × 10^{10} particles/cell) for 180 min in the presence or absence of excess soluble hJAM1. Cells were collected by vacuum filtration, and virus binding was assessed by liquid scintillation.

**Cross-linking Analysis**—Soluble hJAM1 (500 µg/ml) was incubated with a 50-fold molar excess of the water-soluble cross-linking reagent bis(sulfosuccinimidyl) suberate (BS3) in PBS at room temperature for 60 min. Unreacted BS3 was quenched by the addition of 20 mM Tris-HCl (pH 7.5). HeLa cells (1 × 10^9) were incubated with PBS or 2 mM BS3 at 4 °C for 120 min, followed by washing with PBS to remove excess BS3. The efficiency of cross-linking was assessed following lysis of cells in 1% Triton X-100/PBS on ice for 30 min. Lysates were clarified by centrifugation, and hJAM1 was immunoprecipitated using 10 µl hJAM1-specific mAb J10.4. Immunoprecipitates and soluble hJAM1 were resolved by SDS-PAGE. Resolved proteins were transferred to nitrocellulose and immunoblotted with hJAM1-specific antiserum (1:1000). Proteins were detected by ECL. Soluble hJAM1 was tested for the capacity to bind reovirus and compete virus binding in vitro, and HeLa cells were tested for the capacity to bind virus in a radioligand binding assay.

**RESULTS**

**Identification of hJAM1 Domains Required for Reovirus Attachment**—We previously have shown that JAM1 is a serotype-independent reovirus receptor (17). However, specific sequences in JAM1 required for reovirus attachment are not known. To identify domains in JAM1 required for reovirus binding and infection, we generated receptor chimeras using hJAM1 and Ig superfamily relative hCAR (Fig. 1 and Table I). hCAR is incapable of supporting reovirus binding and infection (17) and was selected as the chimera partner for these studies due to its structural similarities to JAM1 (23). A PCR-based approach was used to reciprocally exchange sequences encoding either the D1 or D2 Ig-like domains of wild-type receptor.
Reovirus-JAM1 Binding Analysis

Fig. 2. Reovirus engages the D1 domain of hJAM1. CHO cells were transiently transfected with plasmids encoding the indicated receptor constructs. Following incubation for 24 h to permit receptor expression, cells (1 × 10^6) were stained with hCAR- or hJAM1-specific antisera or adsorbed with reovirus T1L (1 × 10^11 particles). Cell surface expression of receptor constructs and virus binding were assessed by flow cytometry.

cDNAs. We also generated single-domain deletion mutants of hJAM1 to complement data obtained using the chimeric receptor molecules. CHO cells, which lack expression of both JAM1 and CAR (see Refs. 19 and 32 and Fig. 2), were transiently transfected with plasmids encoding wild-type hJAM1 and hCAR, chimeric receptor molecules CJJ and JCJ, and hJAM1 deletion mutants JΔJ and ΔJJ. Cell surface expression of each construct and the capacity of reovirus to bind transfected cells were assessed by flow cytometry (Fig. 2). All constructs were detected at the cell surface. Chimera-transfected cells stained with both hJAM1- and hCAR-specific antisera, indicating that the molecules are indeed chimeric (Fig. 2). Prototype reovirus strain T1L bound cells expressing wild-type hJAM1, chimera JCJ, and deletion mutant JΔJ but failed to bind cells expressing hCAR, chimera CJJ, or deletion mutant ΔJJ (Fig. 2). T3 reovirus strain T3SA—also bound cells expressing constructs that contained the D1 domain of hJAM1 (data not shown). These data demonstrate that both T1 and T3 reoviruses engage the membrane-distal D1 domain of hJAM1.

Reovirus Infection and Growth in CHO Cells Expressing Chimeric and Deletion Mutant Receptors—To determine the role of specific JAM1 domains in reovirus infection, CHO cells were transiently transfected with plasmids encoding the hCAR-hJAM1 chimeras or hJAM1 domain-deletion mutants. Transfected cells were adsorbed with T1L, and the capacity of reovirus to infect these cells was assessed by indirect immunofluorescence. Consistent with the binding experiments, reovirus protein expression was detected after infection of cells expressing wild-type hJAM1, chimera JCJ, and deletion mutant JΔJ (Fig. 3A). As a control, adenovirus infection also was tested by adsorbing transfected cells with Ad 5-ΔF2. Cells expressing hCAR and CJJ supported infection by adenovirus (data not shown), demonstrating that these molecules also can serve as functional virus receptors. To provide further support for the role of hJAM1 D1 in reovirus infection, viral replication was assessed 24 h after adsorption by plaque assay. T1L produced substantially higher yields in cells expressing hJAM1, JCJ, and JΔJ compared with cells expressing receptors that lack the hJAM1 D1 domain (Fig. 3B). Together, these data indicate that the D1 domain of hJAM1 is required for reovirus attachment, infection, and growth.

Competitive Reovirus-hJAM1 Binding Analysis—To assess the capacity of reovirus and hJAM1 to compete for engagement of hJAM1, a series of complementary in vitro and cell binding studies were performed. Due to the apparent high affinity and slow dissociation rate of hJAM1 dimers (23), we first established an experimental system in which equilibrium could be achieved over an extended time course to provide evidence that hJAM1-hJAM1 interactions are saturable and specific and, therefore, support the use of soluble hJAM1 as a competitor for virus binding studies. For these experiments, ELISA plates were coated with 20 nM solutions of soluble hJAM1 ectodomain. This concentration facilitated maximal hJAM1 immobilization (data not shown). Increasing concentrations of biotinylated hJAM1 were then tested for the capacity to bind immobilized hJAM1 in a kinetic binding assay (Fig. 4A). Homophilic hJAM1 interactions occurred in a concentration- and time-dependent manner, approaching equilibrium following incubation for intervals greater than 240 min at 80 and 160 nM concentrations. Furthermore, hJAM1-hJAM1 interactions are specific, since incubation in the presence of 100-fold excess concentrations of unlabeled hJAM1 resulted in no detectable binding following incubation for 180 min (data not shown).

To gather further evidence for the validity of in vitro binding assays for studies of reovirus-hJAM1 interactions, we performed kinetic virus-binding analyses. Binding of biotinylated reovirus T1L to immobilized hJAM1 was assessed over time (Fig. 4B). Reovirus exhibited concentration- and time-dependent binding to hJAM1-coated plates, achieving equilibrium more rapidly than hJAM1 (~180 min) and at lower concentrations (data not shown). Together, these results demonstrate that hJAM1 and reovirus specifically engage immobilized hJAM1 and provide confidence that this in vitro binding assay is a valid method for assessing whether hJAM1 and reovirus compete for overlapping binding sites.

We next used the in vitro binding assay to test the capacity of soluble hJAM1 to compete for reovirus binding to immobilized hJAM1 (Fig. 4C). Biotinylated reovirus T1L particles (2 × 10^11/ml) were mixed with fold molar excess concentrations of soluble hJAM1, calculated to account for 36 copies of α1 per virion particle, and tested for the capacity to bind immobilized hJAM1. Reovirus binding was inhibited in a dose-dependent fashion, with a 50% inhibitory concentration (IC50) between 20-
and 50-fold molar excess soluble hJAM1. At 100-fold molar excess hJAM1, virus binding was reduced to near background levels, which were determined by assessing virus binding in the presence of hJAM1-specific mAb J10.4.

To provide a more complete assessment of reovirus-hJAM1 interactions, we tested the capacity of soluble hJAM1 to compete for reovirus binding to HeLa cells (Fig. 4D). HeLa cells were incubated with radiolabeled T1L virions in the presence of increasing fold molar excess of soluble hJAM1. Similar to results obtained using the in vitro binding assay, reovirus binding to HeLa cells was inhibited by soluble hJAM1 in a dose-dependent fashion, with an IC$_{50}$ between 20- and 50-fold molar excess hJAM1 and nearly complete inhibition by 100-fold molar excess. These data provide additional support for the primary role of hJAM1 in reovirus attachment to cells. Moreover, the large excess of hJAM1 required for competition suggests a higher affinity for reovirus-hJAM1 interactions than those of hJAM1-hJAM1 (35).

Effect of Cross-linking on Reovirus Binding to hJAM1—To more directly assess the importance of residues involved in hJAM1 dimerization for reovirus attachment, we tested the effect of hJAM1 cross-linking on reovirus binding. Soluble hJAM1 was cross-linked by incubation with the water-soluble cross-linking agent BS$^3$. This treatment resulted in the formation of higher order oligomers whose $M_r$ values correspond to hJAM1 dimers (Fig. 5A). Similar results were observed previously for cross-linked mJAM1 (24), suggesting that cross-linking hJAM1 and mJAM1 covalently join the dimeric structures

**FIG. 3.** The D1 domain of hJAM1 is required for reovirus infection and replication. CHO cells were transiently transfected with plasmids encoding the indicated receptor constructs and incubated for 24 h to permit receptor expression. A, transfected cells ($4 \times 10^5$) were infected with reovirus T1L at a multiplicity of infection of 1 fluorescent focus unit/cell and incubated at 37°C for 20 h. Cells were fixed and stained for reovirus protein, and infected cells were identified by indirect immunofluorescence. Representative images are shown. B, transfected cells ($2 \times 10^6$) were adsorbed with reovirus T1L at a multiplicity of infection of 1 plaque-forming unit (PFU)/cell. Reovirus growth was assessed by plaque assay at 0 and 24 h postadsorption. Shown are mean viral titers for three independent experiments. The error bars indicate S.D.
elucidated for both molecules. To assess the effects of cross-linking on reovirus attachment, ELISA plates were coated with 20 nM solutions of either untreated or BS3-treated hJAM1 and tested for the capacity to bind biotinylated T1L virions (Fig. 5B). Cross-linking resulted in a reduction of reovirus binding to immobilized hJAM1 to near background levels, suggesting that reovirus binds a monomeric form of hJAM1 via residues in the dimer interface. To corroborate these results, we tested the capacity of 100-fold excess BS3-treated hJAM1 to compete for reovirus binding to untreated hJAM1 (Fig. 5B). In sharp contrast to untreated hJAM1 at 100-fold molar excess, 100-fold molar excess cross-linked hJAM1 was an inefficient competitor of reovirus binding. These data strongly suggest that the hJAM1 dimer interface is involved in reovirus attachment.

To define the effect of hJAM1 cross-linking on reovirus binding to cells, HeLa cell surface proteins were cross-linked by incubation with BS3. Cross-linked hJAM1 was captured by immunoprecipitation using hJAM1-specific mAb J10.4 and detected by immunoblotting using hJAM1-specific antiserum (Fig. 5C). BS3 treatment of cells resulted in the formation of a very large Mr species (>97 kDa). Following BS3 treatment, the binding capacity of radiolabeled T1L was diminished, with cross-linking resulting in >50% reduction in virus binding (Fig. 5D). Virus binding in the presence of 20 µg/ml hJAM1-specific mAb J10.4 was completely abolished in vitro (Fig. 5B) and on cells (Fig. 5D), indicating that residual binding following BS3 is dependent on hJAM1. Therefore, the consistent reduction in reovirus binding due to cross-linking and the failure of BS3-treated hJAM1 to compete for viral attachment to untreated hJAM1 suggest that reovirus engages hJAM1 using residues within the hJAM1 dimer interface. However, it is also possible that cross-linking alters the conformation of hJAM1 in such a manner to prevent efficient reovirus binding or the cross-linking agent covers a region in the hJAM1 dimer that otherwise would be engaged by reovirus. The apparent inefficiency of cross-linking relative to the effects on virus binding may reflect a loss of antibody-binding epitopes due to BS3 treatment, with resultant diminished band intensity by immunoblotting relative to the untreated species.

Mutational Analysis of Reovirus Binding to hJAM1—To precisely define the region of hJAM1 bound by α1 and to identify residues critical for reovirus attachment, we used a PCR-based approach to generate mutant forms of hJAM1 containing single amino acid substitutions. Guided by the hJAM1 crystal struc-
nature (23), mutations were targeted to solvent-exposed residues in regions of the molecule that are highly conserved between hJAM1 and mJAM1 (Fig. 6 and Table II). Soluble ectodomains of hJAM1 mutants were liberated from glutathione S-transferase-hJAM1 fusion proteins via treatment with thrombin, and 20 nM solutions of the cleavage products were immobilized onto ELISA plates. Biotinylated T1L virions (2.0 x 10^11 particles/ml) were then tested for the capacity to bind the immobilized hJAM1 mutants (Fig. 7). Virus-binding data were normalized for hJAM1 immobilization efficiency, and binding was not detected for any constructs in the presence of 20 µg/ml hJAM1-specific mAb J10.4 (data not shown). Most of the mutations resulted in only minor or modest reductions in virus binding. However, substitution of Ser57 with lysine or Tyr 75 with alanine substantially reduced the efficiency of reovirus binding. Ser57 is a solvent-accessible residue at the apex of hJAM1 adjacent to the dimer interface, and Tyr75 is a core residue at the top of the dimer interface (Fig. 6). Neither of these mutations alters the dimeric nature of the molecule in solution (data not shown), indicating that the observed effects of these mutations on reovirus binding are not attributable to alterations in the capacity of the mutant proteins to form dimers. Thus, these findings suggest that Ser57 and Tyr75 play critical roles in reovirus attachment and highlight the importance of a region at the top of the homodimer interface in reovirus binding to hJAM1.

**DISCUSSION**

Experiments reported here were performed to define the molecular basis of reovirus attachment to hJAM1. The capacity of reovirus to bind, infect, and replicate to high titers in cells expressing chimeric hCAR-hJAM1 receptor constructs and single domain deletion mutants demonstrates that the membrane-distal D1 domain of hJAM1 is an essential component of the reovirus receptor function of hJAM1 (Figs. 2 and 3). Several viruses have been demonstrated to engage cellular receptors via the most distal domain of an Ig superfamily receptor, including adenovirus (hCAR) (16, 36), coxsackievirus (hCAR) (37), human immunodeficiency virus (CD4) (38), measles virus (SLAM) (39), poliovirus (PVR) (40), and rhinovirus (ICAM-1) (41). Thus, utilization of the membrane-distal D1 domain of hJAM1 by reovirus provides additional evidence for a common theme in viral attachment.

Of the virus receptors whose structures have been solved, only adenovirus receptor hCAR and reovirus receptor JAM1 have been demonstrated to form homodimers by contacts between the GFCC-strands of apposing D1 domains (22, 23, 25). Moreover, there are numerous similarities in the attach-
ment proteins of adenovirus and reovirus: 1) both form trimers; 2) both insert into pentamers of capsid proteins at the virion 5-fold symmetry axes; 3) both have fibrous domains formed by unique triple /H9252-spiral structural motifs; and 4) both have globular, virion-distal domains formed by /H9252-barrel structures with unique and identical /H9252-strand connectivity. These similarities suggest functionally convergent evolution of two large, nonenveloped viruses (14). Additionally, the observation that hJAM1 and hCAR share numerous structural and functional similarities leads to the hypothesis that adenovirus and reovirus engage their receptors using similar mechanisms.

Structural and biophysical evidence indicates that adenovirus engages the D1 domain of hCAR using sequences involved in hCAR homodimerization. Specifically, adenovirus fiber knob engages the GFCC face of hCAR (16) in a manner thermodynamically favored over hCAR-hCAR interactions (28). To test the hypothesis that reovirus engages hJAM1 using residues involved in hJAM1 homodimerization, we developed an in vitro binding assay and tested the capacity of hJAM1 to compete for reovirus binding to hJAM1. We first established that hJAM1-hJAM1 and reovirus-hJAM1 interactions are saturable and specific (Fig. 4, A and B). Interestingly, reovirus achieved equilibrium more rapidly in these experiments and at molarities much lower than those of hJAM1 (equilibrium in 180 min at 0.16 nM for reovirus versus 300 min at 80 nM for hJAM1). Limitations of the experimental system do not permit accurate calculations of equilibrium binding constants. Nonetheless, it is likely that reovirus-hJAM1 interactions are thermodynamically favored over hJAM1-hJAM1 interactions.

The calculated K_D of mJAM1 for mJAM1 (~15 nM) (24) approximates that of the purified T3D σ1 head domain for hJAM1 (~60 nM) (17). However, the multivalent nature of σ1

![Fig. 6. Orientation of mutagenized hJAM1 residues.](image)

**Table II**

<table>
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<tr>
<th>Point mutation</th>
<th>Primer Forward</th>
<th>Primer Reverse</th>
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<td>5'-ACGGGGAGATTTAAAGCCCA-3'</td>
</tr>
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<td>S57K</td>
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<td>5'-CAACCGGGTTTATGAAACCA-3'</td>
</tr>
<tr>
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<td>5'-CTTGTATGGCAGCCAAACAG-3'</td>
</tr>
<tr>
<td>T80A</td>
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<td>5'-ATAGGAACGGGCATCTTGT-3'</td>
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<tr>
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<td>5'-ACAGCTTCTCCGCGAGGACCCG-3'</td>
<td>5'-CCGCTTCAGGCGAAGGCTT-3'</td>
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<tr>
<td>T88A</td>
<td>5'-GACCCGGTTGATCTTCTCCCT-3'</td>
<td>5'-TGCGAAAGATGCGACCCCGTC-3'</td>
</tr>
<tr>
<td>T92A</td>
<td>5'-TCTTGTCCGCGGTTATACCC-3'</td>
<td>5'-GCTGTATCCGGCGAAACAG-3'</td>
</tr>
<tr>
<td>S98A</td>
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<td>5'-CCCGTACTGCTTCTGGAAA-3'</td>
</tr>
<tr>
<td>E113K</td>
<td>5'-ATGGTCTCTTAAAGAGGGCCG-3'</td>
<td>5'-GCCGCTTCCTTCTTGAAGACC-3'</td>
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on reovirus particles probably mediates cooperative binding effects that enhance the on rate and stability of reovirus binding to hJAM1. Additionally, soluble hJAM1 competes for reovirus binding to hJAM1 in vitro and on cells with comparable IC_{50} values between 20- and 50-fold molar excess hJAM1 relative to particle-associated σ1 and nearly complete inhibition at 100-fold excess concentrations (Fig. 4, C and D). The relatively high concentration of hJAM1 necessary to detect soluble hJAM1 binding to immobilized hJAM1 precludes the converse competition experiment using reovirus as competitor (100-fold molar excess = 1.3 × 10^{14} particles/ml or 4.8 × 10^{15} copies of σ1/ml). Nonetheless, these data further establish the specificity of reovirus binding to hJAM1 and suggest that reovirus and hJAM1 utilize identical or overlapping sites for attachment and dimerization, respectively. Moreover, the high concentration of hJAM1 necessary to compete reovirus binding provides additional support for virus-hJAM1 associations being thermodynamically favored over homotypic hJAM1 interactions.

To further assess the role of the hJAM1 dimer interface in reovirus binding, we tested the effect of chemically cross-linking hJAM1 on reovirus binding to hJAM1 in vitro and on cells. We found that cross-linking substantially reduced the efficiency of reovirus binding (Fig. 5). Furthermore, cross-linked hJAM1 exhibited only minimal competition for reovirus binding to hJAM1 (Fig. 5B). The most likely interpretation of these data is that access to the hJAM1 dimer interface is required for efficient reovirus binding, although it is also possible that cross-linking induces structural changes in hJAM1 that inhibit viral attachment or that the bound cross-linking reagent prevents access to the hJAM1 dimer. In these experiments, cross-linking HeLa cell surface proteins induced the formation of a very large M_{r} band recognized by antisera specific for hJAM1 following immunoprecipitation with an hJAM1-specific mAb (Fig. 5C). It is possible that formation of a large M_{r} hJAM1-containing species following cross-linking indicates the tight association of hJAM1 with additional cellular proteins that facilitate reovirus entry following attachment to hJAM1 in a manner analogous to the attachment and entry strategy of adenovirus (42). The presence of such proteins also might offer an explanation for the inefficient viral growth in CHO cells observed in the absence of hJAM1 D1 expression (Fig. 3B).

We previously demonstrated that both hJAM1 and mJAM1 function as reovirus receptors (17). Since this observation, two additional human and murine JAM proteins (JAM2 and JAM3) have been identified. However, only JAM1 is capable of supporting reovirus infection (23). Comparative sequence and structural analysis identified regions of conserved residues in hJAM1 and mJAM1, but not hJAM2 or hJAM3, which suggested roles for these conserved regions in reovirus attachment (23). To define residues critical for reovirus binding, mutations were introduced in several solvent-accessible residues covering most of the hJAM1 D1 surface (Fig. 6), and these mutant constructs were tested for the capacity to bind reovirus in vitro. Using this approach, we found that residues Ser{sup 57} and Tyr{sup 75} are important for efficient reovirus binding (Fig. 7). Interestingly, these residues localize to a region at the very top of the hJAM1 dimer interface (Figs. 6 and 8). Mutagenesis of Glu{sup 113}, another residue localized to the top of the dimer interface (Figs. 6 and 8), also diminished reovirus binding, but to a lesser extent than mutations at Ser{sup 57} or Tyr{sup 75} (Fig. 7). In contrast, mutation of the other residues chosen for study had little effect on binding, suggesting that the surface opposite to the dimer interface (the exposed “back” of hJAM1) does not participate in interactions with reovirus.

Of the hJAM1 residues important for reovirus attachment, Ser{sup 57} presents the most accessible potential contact point, and as such, the exposed serine hydroxyl group may provide a hydrogen bond donor for σ1 binding. It is also possible that the S57K mutation introduces a structural change at the top of hJAM1. A lysine side chain could potentially interact with nearby acidic residues Glu{sup 113} and Glu{sup 114}, thereby distorting the reovirus-binding surface. However, this possibility is unlikely, given that mutagenesis of Ser{sup 56} to lysine does not elicit a similar inhibitory effect on reovirus binding. We note that several mutations in a region adjacent to or in the dimer interface (S49A, A51K, S56K, Y83A, and T92A) modestly inhibit binding (Fig. 7). Most of these mutations (Ser{sup 49}, Ala{sup 51}, and Ser{sup 56}) are in close proximity to and form part of the

![Fig. 7. Ser{sup 57} and Tyr{sup 75} facilitate efficient reovirus attachment to hJAM1. ELISA plates were coated with 20 nM solutions of wild-type or point mutant forms of soluble hJAM1. Binding of biotinylated reovirus T1L (2 × 10^{11} particles/ml) to immobilized hJAM1 constructs was assessed after incubation at 37 °C for 180 min. Virus binding was detected following incubation with streptavidin-HRP. The dashed line indicates reovirus binding to wild-type hJAM1. Results are the means of quadruplicate experiments. The error bars indicate S.D. Data are normalized for immobilization efficiency.](downloaded from http://www.jbc.org/)

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contiguous β-strand leading to Ser57. It is conceivable that mutagenesis of some of these residues slightly alters the orientation of the Ser57 side chain in a manner that diminishes the efficiency of reovirus attachment. Residue Tyr75 is buried in the dimer interface. The drastic effect of Y75A on reovirus attachment suggests that disruption of hJAM1 homodimers is required for efficient reovirus binding and, furthermore, that reovirus interacts with residues at the hJAM1 dimer interface. An alternative explanation, given the location of Tyr75 at the hJAM1-hJAM1 interface, might be that the Y75A mutation adversely affects the dimeric structure, thereby preventing reovirus binding to dimeric hJAM1. However, mutant Y75A maintains its dimeric nature in solution (data not shown), making this explanation less likely. Moreover, our competitive binding and cross-linking experiments (Figs. 4 and 5, respectively) strengthen the argument against this second possibility.

Our experiments preclude an exact determination of the mechanism by which Y75A diminishes reovirus binding. Mutagenesis of Tyr75 may alter the dissociation capacity of hJAM1 dimers or remove a potential reovirus contact point. We also consider it possible that reovirus engages hJAM1 using a two-step mechanism that facilitates dimer dissolution and efficient reovirus binding. If so, initial contact with Ser57 may induce a conformational change in reovirus particles, hJAM1, or both molecules to promote the formation of a higher affinity binding state, perhaps by exposing Tyr75. We note that, in the hJAM1 dimer structure, Ser57 is very close to Tyr75 of the apposed hJAM1 monomer (Y75′ in Fig. 8); thus, interaction with Ser57 could easily trigger rearrangements at the JAM1-JAM1 interface. Several lines of evidence lend support to this hypothesis. First, the localization of closely apposed aspartic acid residues in the interior of the α1 head trimer interface suggests that α1 is a metastable protein primed for structural rearrangements (13). Second, although homotypic hJAM1 association cannot be detected in real time by surface plasmon resonance (data not shown), in similar experiments reovirus engages hJAM1 with rapid and saturable kinetics to form a highly stable complex (17). Third, reovirus binding to cell surfaces or cell membrane preparations alters the sensitivity of the virus to proteolysis in a α1-dependent fashion (43), suggesting a role for α1 in particle-associated structural changes. Ongoing biophysical and structural approaches will facilitate a further understanding of the mechanism of reovirus-hJAM1 interactions.

Studies reported in this manuscript provide evidence for possible mechanisms of reovirus binding to hJAM1. They also highlight common mechanisms of attachment for reovirus and adenovirus in which structurally analogous attachment proteins disrupt dimers of structurally analogous receptors to engage and infect target cells. Intriguingly, hJAM1 and hCAR, as well as several other viral receptors, localize to epithelial barriers (44), suggesting that aspects of these physiologic locations provide permissive sites for viral infection. Both hJAM1 and hCAR have been reported to regulate tight junction barrier function (18–20, 45), and adenovirus may usurp this property to facilitate release and spread of progeny virions into the environment (46). Whether reovirus disregulates hJAM1 in a similar manner is not known. It is also possible that hJAM1 and hCAR were simply selected as abundant and convenient attachment moieties that are readily accessible by natural routes of inoculation, which raises the question of why these particular molecules were chosen. An interesting explanation would be that particular receptors associate with additional cellular molecules that contribute to viral replication by facilitating cell entry or activating intracellular signaling to induce a virus-permissive state. Differential requirements for entry or replication may offer an explanation for why reoviruses and adenoviruses have selected distinct molecules for amazingly similar mechanisms of attachment.

Acknowledgments—We thank members of our laboratory for many useful discussions and Jim Chappell and Tim Peters for review of the manuscript. We acknowledge the Nashville Veterans Affairs Hospital Flow Cytometry Facility for assistance and data analysis.

REFERENCES

Reovirus-JAM1 Binding Analysis


