Junctional adhesion molecule-1 (JAM1) is a tight junction-associated immunoglobulin superfamily protein implicated in the regulation of tight junctions and leukocyte transmigration. The structural basis for the function of JAM1 has yet to be determined. Here we provide evidence that JAM1 homodimer formation is important for its function in epithelial cells. Experiments were conducted to determine the effects of a panel of JAM1 monoclonal antibodies on epithelial barrier recovery after transient disruption by calcium switch. Two monoclonal antibodies were observed to inhibit barrier recovery in contrast to another monoclonal antibody that had no effect. Epitope mapping by phage display revealed that both inhibitory antibodies bind to a region of JAM1 located within the N-terminal Ig-like loop (residues 111–123). Competition experiments with synthetic peptides and site-directed mutagenesis confirmed the location of this epitope. Analysis of the crystal structure of JAM1 revealed that this epitope includes residues within the putative homodimer interface, and one of the two inhibitory antibodies was then shown to block JAM1 homodimer formation in vitro. Finally, mutations within the homodimer interface were shown to prevent enrichment of JAM1 at points of cell contact, presumably by interference with homophilic interactions. These findings suggest that homodimer formation may be important for localization of JAM1 at tight junctions and for regulation of epithelial barrier function.

A crucial function of epithelial cells is in the maintenance of a selective barrier separating the external from internal environments. It is well known that tight junctions (TJs) play an important role in the maintenance and regulation of these barriers. Located at the apical-most part of the lateral epithelial cell membrane, TJs regulate paracellular diffusion of ions and small molecules across epithelial barriers. In addition, TJs serve as a fence between the apical and basolateral domains of polarized cells and facilitate bi-directional signal transduction between the intracellular and extracellular environments. TJs are composed of both transmembrane and cytoplasmic structural elements. Transmembrane protein members include occludins, claudins, coxsackie adenovirus receptors, and junctional adhesion molecules (JAMs). Knowledge of how these various proteins contribute to TJ function is continually evolving.

JAM1 is an immunoglobulin superfamily protein that localizes to TJs of epithelial and endothelial cells (1) as well as to the surface of leukocytes (2). Evidence suggests a role for JAM1 in TJ assembly (3). Exogenous expression of JAM1 in CHO cells has been shown to promote localization of ZO-1 and occludin at points of cell contact (4). Monoclonal antibodies to JAM1 have been shown to inhibit reassembly of TJs and block recovery of transepithelial resistance to ion flux (3). Similar effects on epithelial barrier function were observed with recombinant soluble JAM1 consisting of the extracellular domain fused to IgG Fc (5). In addition to TJ assembly, there is evidence to suggest that JAM1 may play a role in regulating leukocyte transepithelial migration in murine models (1, 6, 7).

JAM1 is 36–40-kDa immunoglobulin superfamily protein consisting of an extracellular domain, a single transmembrane domain, and a short cytoplasmic tail (1). The extracellular domain of JAM1 contains two Ig-like loops, the proximal membrane of which contains two N-glycosylation sites (1). The cytoplasmic tail of JAM1 contains a PDZ-binding motif putatively involved in the binding of JAM1 to the cytoplasmic Td-associated protein ZO-1 (8, 9). Three homologs to JAM1 have been reported as follows: JAM-2, which is expressed primarily on endothelial cells (10, 11); JAM-3, which is expressed on leukocytes (12–14); and JAM-4, which was recently reported in kidney and intestinal epithelia (15).

The structural basis for the role of JAM1 in regulation of epithelial and endothelial barriers is largely unknown. Evidence suggests that homophilic interactions are important for JAM1 function. The crystal structure of JAM1 predicts that it forms stable homodimers (16, 17). Cross-linking experiments suggest that JAM1 forms homodimers in solution and on the surface of transfected CHO cells (4). Other studies suggest that heterotypic interactions are important for JAM1 function. For example, Ostermann et al. (6) observed that LFA-1 (α<sub>β</sub>) interactions are important for T cell binding and migration, and Naik et al. (18) suggest that JAM1 binds to α<sub>β</sub><sub>2</sub>. Together these results suggest that JAM1 function in vivo may involve multiple types of protein-protein interactions, perhaps both homotypic and heterotypic in nature.

Here we present evidence that homophilic JAM1 interactions are important for regulation of barrier function in epithelial cells. We demonstrate the following: (i) monoclonal JAM1 antibodies that inhibit resealing of epithelial monolayers bind to a common region within the extracellular domain of JAM1; (ii) this functional epitope is located within the N-terminal Ig-like loop (residues 111–123); (iii) residues within the putative ho-
modem interfacial contact to this functional epitope; (iv) an inhibitory JAM1 antibody that binds to this epitope blocks JAM1 homodimer formation in vitro; and (v) homodimer interfa-

cient mutants fail to concentrate at points of contact between transcellular

EXPERIMENTAL PROCEDURES

Cell Culture—COS-7 cells (ATCC) and SK-CO15 colonic epithelial cells (19) were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% FBS, 2 mM L-glutamine, 100 IU penicillin, 100 μg/mL streptomycin, 15 mM HEPES, and 1% non-essential amino acids (Celgro). Cells were subcultured and harvested with 0.05% trypsin and 5 mM EDTA to make single cell’s balanced salt solution. For transepithelial resistance recovery experiments, SK-CO15 cells were grown on collagen-coated polycarbonate membranes with 0.4-μm pore size and 0.33-cm² surface area (Costar).

Recombinant Protein Production—JAM1-AP was produced in our laboratory as described previously (3). Briefly, a DNA fragment encoding the putative extracellular domain of human JAM1 was cloned into the AP-tag2 vector that encodes the catalytic domain of human placental alkaline phosphatase at the C terminus. The vector was then tran-
siently transfected into COS-7 cells using DEAE-dextran. Secretion of JAM1-AP was monitored by detection of alkaline phosphatase activity in the culture medium. JAM1-AP was affinity purified on an anti-AP column (Amersham) and eluted with 100 mM triethylamine, pH 11.5, and neutralized with 100 mM HEPES, pH 5. Fractions were combined and dialyzed in HEPES-buffered saline for 16 h at 4 °C. Purity was confirmed by SDS-PAGE. The mutant JAM1-AP-N117K was produced using the GeneTaal site-directed mutagenesis kit (In-vitrogen). Recombinant JAM1-AP-N117K was produced in COS-7 cells as described above. Mutant protein production was verified by assaying the culture medium for alkaline phosphatase activity and by reactivity with JAM1 mAb 1H2A9. For homodimerization assays, JAM1 was produced in bacteria as reported previously (17). Dr. Terence Dermody kindly provided a plasmid encoding GST fused to the extracellular domain of JAM1 (GST-JAM1). GST-JAM1 was expressed in BL21 Escherichia coli and purified by affinity chromatography with glutathione-Sepharose (BD Biosciences). The extracellular domain of JAM1 was cleaved with thrombin (Sigma), and the thrombin was then adsorbed onto benzamidine-Sepharose (Amersham Biosciences). Purity of the JAM1 protein was assessed by SDS-PAGE. For biontinylation, NHS-LS-biotin (Pierce) was reacted with purified JAM1 according to the manufac-

ABSTRACT—JAM1, a member of the Ig superfamily, is thought to function as a homodimeric transmembrane protein that participates in tight junction (TJ) barrier function. To disrupt intercellular junctions, cells were treated calcium-free medium, transepithelial resistance was recorded for each monolayer yielding TER values less than 50 ohms/cm². Cells were washed twice with normal growth medium and incubated in normal growth medium containing 10 μg/mL of antibody J3F.1, J10.4, 1H2A9, mouse IgG1, or J10.4 and 1H2A9, mouse IgG1 for 1 h at 37 °C. After 1 h, wells were washed, and a solution containing 1 mM 2,2’-azobis(3-ethylbenzothiazoline-6-sulfonic acid and 10 mM H₂O₂ in 100 mM citrate buffer, pH 4.2, was added. Binding of biotin-J10.4 was measured indirectly by absorbance at 405 nm in a microtiter plate reader (Molecular Devices).

For mutant JAM1 protein binding experiments, wells were coated with mAbs J3F.1, J10.4, and 1H2A9 at 10 μg/mL for 16 h at 4 °C. Non-specific protein binding was blocked by incubation of wells with 5% delipidated milk in HEPES-buffered saline for 1 h at 37 °C. Peptides were dissolved in MeSO and then serially diluted in blocking buffer containing 0.4 μg/mL JAM1-AP. After incubation for 1 h at 37 °C, wells were washed 3 times with HEPES-buffered saline, and a solution containing 1 mg/mL p-nitrophenyl phosphate (Sigma) in 0.2 M Tris was added. Alkaline phosphatase activity was then determined by absorbance at 405 nm in a microtiter plate reader.

For homodimerization experiments, a published assay (4) was mod-

fied to test the effects of anti-JAM1 mAbs on JAM1 homobinding. Wells were coated with JAM1 at 12.5 μg/mL for 16 h at 4 °C. Non-specific protein binding was blocked by incubation with 2% BSA plus 0.05% Tween 20 in phosphate-buffered saline. Fab fragments of J10.4, 1H2A9, or control antibody were serially diluted in blocking buffer containing biotinylated JAM1 (10 μg/mL) and incubated for 1 h at 37 °C. Wells were washed 3 times with 0.2% BSA and 0.05% Tween 20 in phosphate-buffered saline and then incubated with horseradish peroxidase-streptavidin (1 μg/mL) for 30 min at 37 °C. As described above, 2,2’-azobis(3-ethylbenzothiazoline-6-sulfonic acid substrate was added, and biotin-JAM1 binding was quantified by absorbance at 405 nm.

Epitope Mapping by Phage Display—To display the epitopes of JAM1 mAbs, we used random peptide phage libraries LL9 and CL10, which were previously produced in our laboratory (21). Oligonucleotides encoding random peptides were cloned into the pIII gene of M13K8X9. The LL9 library contains random nonapeptides with a diversity of 3.66 × 10⁹ unique clones. The CL10 library contains random cysteine-

The LL9 library contains random nonapeptides with a diversity of 3.66 × 10⁹ unique clones. The CL10 library contains random cysteine-

containing decapeptides with a diversity of 1.04 × 10¹⁰ unique clones. The LL9 and CL10 libraries were panned in HEPES-buffered saline (1). The titer of the crude and purified phage was determined by plaque assay to estimate the number of viral particles in each phage display library. Peptides were commercially synthesized and pur-

ified by high pressure liquid chromatography to >90% purity (Emory Biomedical), and for the drug screen, the purified peptides, dissolved in saline, were oxidized. All peptides were analyzed by matrix-assisted laser desorption ionization/time of flight to confirm purity and oxidation state.

Crystal Structure Analysis—The atomic coordinates for the crystal structure of human JAM1 were downloaded from the Protein Data Bank (PDB code 1MRQ). Analysis was performed using Swiss-PdbViewer/Deep View version 3.6 (GlauxoSmithKline).

Production and Localization of JAM-1 Mutants—To produce JAM1 mutants, DNA encoding full-length JAM1 was cloned into pIRE52-EGFP (Clontech), and mutations were made using the QuickChange kit.
Involvement of JAM1 Homodimer in Barrier Function

**RESULTS**

**Differing Effects of JAM1 Antibodies on Barrier Recovery**—Experiments were conducted to determine the effects of a panel of JAM1 antibodies on epithelial barrier recovery after transient disruption of TJJs by calcium switch. Confluent SK-CO15 colonic epithelial monolayers were treated with calcium-free medium for 45 min to disrupt intercellular junctions. TER values were monitored for each monolayer and were consistently observed to decrease from values greater than 1000 ohm-cm² to values less than 50 ohm-cm² in response to calcium depletion. In accordance with decreases in TER, immunofluorescence staining of TJAs revealed marked disruption of the characteristic “chicken wire” staining patterns of occludin, JAM1, and ZO1 (data not shown). At this point, cultures were returned to normal growth medium that was supplemented with anti-JAM1 mAbs J3F.1, J10.4 or 1H2A9 or with control IgG. Transepithelial resistance to passive ion flux was monitored at 45 min to disrupt intercellular junctions. TER experiments were conducted to determine whether inhibitory antibodies J3F.1 and J10.4 act on barrier recovery. To control for nonspecific effects of antibody fragmentation, 1H2A9-Fab was also tested. As with whole 1H2A9 IgG, no effect on barrier recovery was observed for 1H2A9-Fab (data not shown).

To determine whether the effects of J3F.1 and J10.4 on barrier recovery are attributable to antibody-mediated cross-linking, additional experiments were conducted using Fab fragments. As illustrated in Fig. 1, cultures treated with J10.4-Fab only recovered 13% after 22 h as compared with controls that recovered 100%. This result is comparable with the effect observed with intact mAb J10.4 and suggests that antibody-mediated cross-linking is not responsible for the effect of J10.4 on barrier recovery. To control for nonspecific effects of antibody fragmentation, 1H2A9-Fab was also tested. As with whole 1H2A9 IgG, no effect on barrier recovery was observed for 1H2A9-Fab (data not shown).

Because of the marked functional differences observed between antibodies J3F.1, J10.4, and 1H2A9, we carefully assessed the epithelial cell staining patterns of these antibodies using direct immunofluorescence. The staining patterns of all three antibodies were identical (not shown). Thus, it is unlikely that the inhibitory effects of J3F.1 and J10.4 are due to cross-reactivity with other proteins or due to differential affinity for specific subpopulations of JAM1.

**J3F.1 and J10.4 Compete for Binding to JAM1**—Experiments were conducted to determine whether inhibitory antibodies J3F.1 and J10.4 bind to a common region of JAM1. Competition assays were performed in which binding of biotinylated J10.4 to immobilized JAM1 was measured in the presence of antibodies J3F.1, J10.4, 1H2A9, control IgG1, or no antibody. As seen in Fig. 2, control IgG1 did not affect binding of biotin-J10.4 to JAM1. Antibody J3F.1, however, completely abrogated binding of biotin-J10.4, and this effect was comparable with that observed with excess unlabeled J10.4. The reduction in binding was comparable with the background binding measured without labeled J10.4 present (data not shown). In similar experiments, binding of labeled J3F.1 to JAM1 was blocked by J10.4 (data not shown). These results suggest that J3F.1 and J10.4 compete for epitopes on JAM1 that are either common or in close proximity. In contrast to the competition observed between J3F.1 and J10.4, antibody 1H2A9 did not block binding of either J10.4 (Fig. 2) or J3F.1 (not shown). These results suggest that 1H2A9 binds to an unrelated epitope on JAM1 sufficiently distant from the epitopes of J3F.1 and J10.4 to avoid steric hindrance from these antibodies.

**Epitope Mapping by Phage Display**—Random peptide phage display was used to map the epitopes of JAM1 antibodies J3F.1 and J10.4.
Two phage libraries, a linear nonapeptide library (LL9) and a constrained decapeptide library (CL10), were screened to identify peptides that bind to antibodies J3F.1 and J10.4. After three rounds of immunoaffinity selection, an increase in phage titer of $10^4$ was observed for phage selected by J10.4 from libraries CL10 and LL9 (data not shown). An increase in phage titer of $10^4$ was also observed for phage selected by J3F.1 from the CL10 library, but no increase in titer was observed with J3F.1 and library LL9.

The identities of phage-displayed peptides selected by J3F.1 and J10.4 were determined by DNA sequencing. As illustrated in Fig. 3, analysis of peptides selected by both J3F.1 and J10.4 revealed a consensus sequence resembling $\text{VSEEGGNSYGVEK}$ of the membrane-distal Ig loop on the extracellular domain of JAM1. The most frequent motif was $\text{G(G/S)N}$, which was present in all but one peptide, and several peptides contained the sequence $\text{GGN}$, which matched exactly to $\text{GGN}$ of JAM1. Several peptides also contained residues with similarity to $\text{VSEE}$, $\text{SY}$, and $\text{EVK}$, but these motifs appeared less frequently than the $\text{GGN}$ motif.

Although many similarities were noted among peptides selected by J3F.1 and J10.4, some differences were noted as well. As shown in Fig. 3, antibody J10.4 selected peptides with residues resembling $\text{EVK}$ of JAM1, but no phage selected by J3F.1 contained this motif. In addition, J3F.1 only selected phage from the constrained CL10 library, although J10.4 selected phage from both the constrained CL10 library and the linear LL9 library. These results suggest that epitopes of J3F.1 and J10.4 are not identical but have similar amino acid composition and are spatially related.

### Peptides Block J3F.1 and J10.4 Binding to JAM1

To determine whether the peptides selected by phage display mimic an actual epitope of JAM1, competition assays were performed. Synthetic peptides corresponding to phage-displayed sequences were tested for their ability to block JAM1 binding to mAbs J3F.1 and J10.4. The characteristics of these peptides are summarized in Table I. As illustrated in Fig. 4, synthetic peptides JRP1, JRP2, and JRP3 produced concentration-dependent decreases in JAM1 binding to J3F.1 and J10.4. The most potent peptide, JRP1, blocked JAM1 binding to J3F.1 with an IC$_{50}$ of 50 nM, whereas JRP2 blocked binding of JAM1 to J3F.1 with an IC$_{50}$ of 1 mM. Although JRP3 decreased JAM1 binding to J10.4, 50% inhibition was not reached at concentrations as high as 3 mM. Overall, the competition observed between JAM1 and JRP1, JRP2, and JRP3 for binding to mAbs J3F.1 and J10.4 suggests that these peptides mimic specific epitopes on JAM1.

To investigate whether the observed effects of peptides JRP1, JRP2, and JRP3 could be due to nonspecific factors, control peptides were synthesized and tested for their ability to inhibit JAM1 binding to J3F.1 and J10.4. The control peptide CTL1 was designed as a scrambled version of JRP1 with flanking cysteine residues, and the control peptide CTL2 consisted of a random amino acid sequence. Neither control peptide affected JAM1 binding at any concentration tested up to 3 mM (Fig. 4). These results suggest that the observed inhibition of JAM1 binding by phage-derived peptides is not due to nonspecific factors common to all peptides.

To complement the results obtained with phage-derived peptide sequences, competition experiments were performed using...
a peptide derived from the primary sequence of JAM1. This peptide, JAM110 (Table I), was designed based on the phage display consensus (Fig. 3) implicating 111VSEEGGNSYGEVK123 of JAM1. As illustrated in Fig. 4, peptide JAM110 inhibited binding to J10.4 in a concentration-dependent manner with a maximum inhibition of 25% at 3 mM. A similar effect was observed for this peptide when tested for its ability to block JAM1 binding to J3F.1 (not shown). These results are consistent with data suggesting that J3F.1 and J10.4 bind to a common epitope that may involve 111VSEEGGNSYGEVK123 of JAM1.

Point Mutation Blocks Binding of J3F.1 and J10.4 to JAM1—

To test the possibility of involvement of 111VSEEGGNSYGEVK123 in JAM1 binding to J3F.1 and J10.4, site-directed mutagenesis was performed. Given the strong consensus for asparagine 117 among phage selected by J3F.1 and J10.4 (Fig. 3) and the solvent exposure of this residue as predicted by the crystal structure (Fig. 6), asparagine 117 was selected for mutagenesis. A lysine substitution was made at this position yielding the mutant protein JAM1-N117K. Experiments were performed using recombinant protein to determine the effect of this mutation on JAM1 binding to mAbs J3F.1, J10.4, and 1H2A9. In each case, the results were compared with binding of wild type JAM1. As illustrated in Fig. 5, the N117K mutation completely abrogated binding of JAM1 to J3F.1 and J10.4. It did not, however, affect binding to 1H2A9. These results support the conclusion that J3F.1 and J10.4 bind to a region of JAM1 that includes Asn-117 and that 1H2A9 binds to a different region of the protein.

### Table I

| Peptide  | Sequence             | Origin       | Antibody | IC$_{50}$ $^b$ (μM) |
|----------|----------------------|--------------|----------|---------------------|
| JRP1     | CQPWDVGSNKEC         | Phage-derived| J3F.1    | 50                  |
| JRP2     | CPGENRLWGYLC         | Phage-derived| J3F.1    | 1000                |
| JRP3     | ANWGWGNSE            | Phage-derived| J10.4    | >3000               |
| JAM110   | MVSEEGGNSYGE         | JAM1 sequence|          | >3000               |
| CTL1     | CEQKPNWSDGVC         | JRP1 scramble|          | NI $^c$             |
| CTL2     | EQNTRPAYCD           | Random sequence|          | NI                  |

$^a$ Selection antibody reported only for sequences generated by phage display.
$^b$ IC$_{50}$ values represent the concentration at which each peptide reduced binding of JAM1 to J3F.1 or J10.4 by 50%.
$^c$ No concentration-dependent inhibition of JAM1 binding was observed.

![Peptide Concentration (mM)](image)

Fig. 4. **Peptides compete with JAM1 for binding to J3F.1 and J10.4.** Synthetic peptides were serially diluted in blocking buffer containing JAM1-AP and incubated in microtiter wells coated with either J3F.1 or J10.4. Alkaline phosphatase activity of bound JAM1-AP was measured by absorbance at 405 nm. As detailed in Table I, peptides JRP1 (■), JRP2 (○), and JRP3 (●) are JAM1-related peptides derived from sequences selected by phage display. Peptide JAM110 (×) corresponds to residues 110–121 of JAM1. Peptide CTL1 (□) is a cysteine-constrained, scrambled version of JRP1. The sequence of peptide CTL2 (○) was selected at random. The sequences of these peptides are listed in Table I.

![Bound JAM1 (OD405)](image)

**Fig. 5.** **Mutation N117K abrogates binding of JAM1 to J3F.1 and J10.4.** Asparagine 117 of JAM1 was substituted with a lysine residue by site-directed mutagenesis. Recombinant JAM1-AP and JAM1-N117K-AP proteins were expressed in COS-7 cells, and cell culture supernatant containing secreted protein was incubated in microtiter wells coated with J3F.1, J10.4, or 1H2A9. Protein binding was assayed by alkaline phosphatase activity in each well.
Molecular Model of the J3F.1 and J10.4 Epitope—The crystal structure of the extracellular domain of human JAM1 (17) was used to model the putative epitopes of J3F.1 and J10.4. Fig. 6 illustrates that the extracellular domain of JAM1 consists of two Ig-like domains, designated D1 and D2. The N-terminal D1 domain is formed by two antiparallel $\beta$-sheets consisting of strands AB EF and GF CC'C'. According to the crystal structure, interactions between the GFCC'C' faces of D1 domains of adjacent JAM1 molecules facilitate formation of stable homodimers (17). This model of JAM1 homodimer formation was used to investigate the structural basis for the inhibitory effects of J3F.1 and J10.4 on epithelial barrier formation.

Our phage display and mutagenesis data suggest that J3F.1 and J10.4 bind to residues included within $^{111}$VSEEGGNSYGEVK$^{123}$ of JAM1 (Figs. 3–5). Analysis of the primary structure of JAM1 reveals that these residues are located within $\beta$-strands F and G of domain D1 (Fig. 6B). These same strands form part of the $\beta$-sheet involved in the homodimer interface (17). In fact, the crystal structure predicts that residues Glu-114, Tyr-119, and Glu-121 are specifically involved homophilic interactions between adjacent JAM1 molecules (17). Fig. 6A illustrates the dramatic spatial proximity of the $^{111}$VSEEGNGSYGEVK$^{123}$ epitope and the homodimer interface. Nine critical residues were reported to be directly involved in homophilic interactions (17), and all nine of these residues are either located within $^{111}$VSEEGNGSYGEVK$^{123}$ or immediately adjacent to it. Together, these results suggest that the epitope of J3F.1 and J10.4 overlaps with the homodimer interface.

J10.4 Fab Fragments Disrupt JAM1 Homodimer Formation—Having observed that mAb J10.4 blocks epithelial barrier recovery (Fig. 1) and may bind to a region of JAM1 implicated in homodimer formation (Fig. 6), experiments were conducted to investigate whether mAb J10.4 can disrupt JAM1 homodimer formation in vitro. Competition assays were performed in which binding of biotin-JAM1 to immobilized JAM1 was measured in the presence of varying concentrations of Fab fragments of J10.4 or control antibody. As illustrated in Fig. 7, J10.4-Fab produced a concentration-dependent decrease in JAM1 homophilic binding. Maximal inhibition of JAM1 homophilic binding was observed at 50 $\mu$g/ml, with an IC$\text{}_{50}$ occurring at 3 $\mu$g/ml for J10.4-Fab. In contrast, control Fab did not affect JAM1 homophilic binding at any concentration up to 50 $\mu$g/ml (Fig. 7). These results are consistent with the structural data (Fig. 6) suggesting that J10.4 binds to a region of JAM1 important for homodimer formation. Given the observation that antibody J10.4 blocks epithelial barrier recovery (Fig. 1), these data support the hypothesis that homodimer formation is important for JAM1 function in epithelial tight junctions.

Interestingly, antibody 1H2A9 was also tested for its ability to disrupt JAM1 homodimer formation. Although a concentration-dependent decrease in homophilic JAM1 binding was observed for 1H2A9-Fab, it was 3-fold less potent and 20% less efficacious than J10.4 (data not shown).

**JAM1 Homodimer Interface Mutants Exhibit Diffuse Membranous Staining and Do Not Concentrate at Junctions between Transfected Cells**—Previous studies (1) have shown that JAM1 localizes at points of contact in transfected CHO cells. In mixed cultures, however, JAM1 was not observed to localize at points of contact with non-transfected cells (1). Investigators hypothesized that homophilic interactions between JAM1 on adjacent cells stabilizes its localization at points of contact (1, 4). Experiments were therefore conducted to investigate the effect of mutations in the homodimer interface on the localization of JAM1 in transfected CHO cells. These mutations included deletion of the entire N-terminal Ig-like loop, DL1, and substitutions at two residues implicated in salt bridge formation within the homodimer interface, E61R/K63E (17). In addition, the N117K mutation, which was observed to block J3F.1/J10.4 binding (Fig. 5), was also tested for its effect on JAM1 localization.

As illustrated in Fig. 8A, wild type JAM1 localized specifically at points of contact between transfected cells but not at boundaries with non-transfected cells or where there were no adjacent...
Involvement of JAM1 Homodimer in Barrier Function

Fig. 7. J10.4-Fab blocks JAM1 homophilic interactions. Fab fragments of anti-JAM1 mAb J10.4 were serially diluted in blocking buffer containing 10 µg/ml biotin-JAM1 and incubated in wells coated with JAM1. Binding of biotin-JAM1 was assessed by incubation with streptavidin-horseradish peroxidase and measurement of absorbance at 405 nm in the presence of substrate. Control Fab fragments were tested under the same conditions.

Fig. 8. JAM1 homodimer interface mutants fail to concentrate at junctions between transfected cells. CHO cells were transfected with DNA encoding wild type JAM1 and mutants predicted to disrupt the homodimer interface. Localization of JAM1 and mutants was assessed by immunofluorescent confocal microscopy. A, wild type JAM1 localizes exclusively at boundaries with transfected cells but not at boundaries with non-transfected cells or areas of the cell membrane that do not contact cells at all. B, mutation N117K, which blocks J3F.1/J10.4 binding, does not affect enrichment of JAM1 at points of cell contact. C and D, homodimer interface mutants lacking the entire N-terminal Ig-like loop, DL1, or containing mutations at critical residues, E61R/K63E, demonstrate diffuse membranous staining, even at points that do not contact transfected cells.

Discussion

Here we report identification of a functional epitope on JAM1 that has a role in regulation of epithelial barrier function. We have shown that JAM1 monoclonal antibodies J3F.1 and J10.4 inhibit epithelial barrier recovery in contrast to another JAM1-specific mAb 1H2A9 that does not (Fig. 1). Antibody competition experiments (Fig. 2) and the results of epitope mapping by phage display (Fig. 3) suggest that inhibitory mAbs J3F.1 and J10.4 bind to a common region of JAM1 involving 111VSEE-GGNSYGEVK123. Peptide competition (Fig. 4) and mutant binding experiments (Fig. 5) support this conclusion. Analysis of the crystal structure of JAM1 reveals the location of this epitope to be within the F and G strands of the N-terminal D1 domain, inclusive of and adjacent to residues that compose the putative homodimer interface (Fig. 6). Inhibitory mAb J10.4 was shown to block JAM1 homophilic interactions in competitive binding assays (Fig. 7), and mutations within the homodimer interface were observed to affect localization of JAM1 at points of cell contact (Fig. 8). Together these results suggest the importance of JAM1 homodimer formation in epithelial barrier function.

Differing Effects of JAM1 mAbs on Barrier Function—As illustrated in Fig. 1, JAM1 mAbs J3F.1 and J10.4 blocked barrier recovery of epithelial monolayers, which contrasts with the lack of effect observed for mAb 1H2A9. Although it is possible that this contrast is due to differences in affinity for JAM1, ELISA data suggest that the affinities of these antibodies for JAM1 are comparable (data not shown). The observation that 1H2A9 binds to JAM1 without affecting barrier recovery therefore suggests that 1H2A9 binds to an epitope of JAM1 that is not critical for its function in TJ.s. Furthermore, the lack of efficacy of 1H2A9 also argues against the notion that the effects of J3F.1 and J10.4 are nonspecific effects related to antibody binding to a TJ-associated protein. Finally, it is important to consider the possibility that antibody-mediated cross-linking contributes to the effects of J3F.1 and J10.4 on barrier function, but the observation that Fab fragments of J10.4 block barrier recovery (Fig. 1) discounts this possibility. Overall, the differing effects of J3F.1, J10.4, and 1H2A9 suggest that specific regions on the extracellular domain of JAM1 are critical for regulation of epithelial barrier function.

Epitope Mapping by Phage Display—Phage-displayed peptides bearing affinity for J3F.1 and J10.4 were immunofinity-selected, and the identity of each peptide was determined by DNA sequencing. A strong consensus was observed for 117GGN117 of JAM1 among phage selected by both J3F.1 and J10.4. Every phage sequenced contained an asparagine at the position corresponding to Asn-117. Although a less dramatic consensus was observed for 111VSEE114 and 118SYGEVK123, these data suggest that other residues around 117GGN117 contribute to binding of J3F.1 and J10.4 to JAM1.

Although the antibody competition data (Fig. 2) and phage display data (Fig. 3) suggest that J3F.1 and J10.4 bind to related regions of JAM1, this does not prove that the epitopes are identical. In fact, our phage display data suggest that the same differences may exist between the J3F.1 and J10.4 epitopes with regard to amino acid composition and structural conformation. For example, J3F.1 panning experiments selected peptides from only the CL10 library, whereas J10.4 selected peptides from both the CL10 and LL9 libraries. Further experiments are needed to determine whether the epitopes are identical.

cells at all. In contrast, both the deletion mutant DL1 (Fig. 8C) and the salt bridge mutant E61R/K63E (Fig. 8D) stained the entire circumference of the cell, even at boundaries with non-transfected cells and in areas where there were no adjacent cells at all. These results suggest that disruption of the homodimer interface prevents JAM1 from concentrating at junctions with other transfected cells. Interestingly, the N117K mutation (Fig. 8B), which was shown to block J3F.1/J10.4 binding to JAM1, was not observed to affect JAM1 localization at cell contacts (Fig. 8B) as compared with wild type JAM1 (Fig. 8A). To verify that expression levels of mutant and wild type JAM1 were equivalent, lysates from mutant- and wild type-transfected cells were immunoblotted for JAM1, and no differences were observed (not shown). Thus, it is unlikely that differences in exogenous protein levels contributed to the differences in localization observed for the homodimer interface mutants.
thermore, J3F.1 did not select peptides with the motif EL(K/R) as J10.4 did. Overall, the consensus among phage selected by antibodies J3F.1 and J10.4 was quite similar, but subtle differences were noted. These observations suggest that J3F.1 and J10.4 bind to closely related but not identical regions on JAM1.

Peptide Competition with JAM1—Synthetic peptides corresponding to peptides selected by phage display were observed to compete with JAM1 for binding to J3F.1 and J10.4 (Fig. 4), suggesting that these phage display peptides mimic antibody-binding sites on JAM1. In addition, a synthetic peptide derived from the primary sequence of JAM1 (residues 110–121) was also observed to compete with JAM1 for binding to J10.4 (Fig. 4) and J3F.1 (data not shown), which is consistent with the notion that some of the residues 111–123 are involved in binding of both J3F.1 and J10.4 to JAM1.

Differences in potency were noted among synthetic peptides with respect to their abilities to block JAM1 binding. The most potent were peptides JRP1 and JRP2 with IC50 values of 50 μM and 1 mM, respectively, and the least potent were peptides JRP3 and JAM110, having IC50 values above 3 mM. The differences in affinity of these peptides is likely due to the fact that JRP1 and JRP2 contain flanking cysteines that form disulfide bridges. Such constraints are likely to stabilize JRP1 and JRP2 in conformations that are more conducive to binding J3F.1 and J10.4. In fact, the crystal structure of JAM1 predicts that residues 111–123 form a hairpin turn between two adjacent β-strands that may be better represented by a structurally constrained peptide than a linear peptide. Thus, additional energy would likely be required for peptides JRP3 and JAM110 to fold into the conformation needed to bind J3F.1 and J10.4.

Effect of Mutation Asn-117 on JAM1 Binding to Antibodies—Mutation N117K in JAM1 was observed to block binding of monoclonal antibodies J3F.1 and J10.4 (Fig. 5). This mutation did not affect binding of 1H2A9 to JAM1 (Fig. 5). These results suggest that J3F.1 and J10.4 both bind to a region of JAM1 that includes N117K and that 1H2A9 does not bind to this same region. The phage consensus data (Fig. 3) and peptide competition data (Fig. 4) are in agreement with these results and support the notion that the region of JAM1 containing residues 111–123 is involved in binding to J3F.1 and J10.4. Although it is possible that mutation of Asn-117 alters the structural conformation of JAM1 in a manner that affects antibody binding, the fact that 1H2A9 binds to N117K as well as to the wild type protein argues against this possibility. In addition, the crystal structure of JAM1 suggests that the side chain of Asn-117 is solvent-exposed and oriented such that the N117K substitution should not greatly affect the overall conformation of JAM1 (data not shown). Overall, the mutagenesis data are consistent with the phage display data and support a role of the region of JAM1 containing residues 111–123 in binding of J3F.1 and J10.4.

Structural Analysis of J3F.1/J10.4 Epitope—Given the results of phage display and mutagenesis experiments, we examined the location of residues 111–123 within the human crystal structure of JAM1 (Fig. 6). Residues 111–123 form part of β-strands F and G of domain D1 of JAM1, and Asn-117 is situated on the turn between these two strands. According to the crystal structure, residues 114, 119, and 121 are directly involved in the JAM1 homodimer interface (17). These findings suggest that residues within the putative homodimer interface contribute to the functional epitope defined by inhibitory mAbs J3F.1 and J10.4. Furthermore, these results support the hypothesis that homodimer formation is an important structural motif for JAM1 function in epithelial cells.

Effect of J10.4 on JAM1 Homophilic Interactions—The observation that J10.4-Fab blocked JAM1 homophilic binding in vitro (Fig. 7) supports structural epitope mapping data that suggest that the J3F.1/J10.4 epitope includes part of the JAM1 homodimer interface (Fig. 6). Given that J10.4 and J3F.1 were shown to block epithelial barrier recovery (Fig. 1), these data also suggest that homodimer formation may be important for JAM1 function in epithelial cells. We note, however, that differences do exist between the in vitro conditions presented in Fig. 7 and the in vivo conditions presented in Fig. 1, i.e., the homophilic interactions assay was conducted in a pure system using recombinant protein consisting of only the extracellular domain of JAM1. In contrast, the barrier recovery assay was performed using full-length JAM1 expressed on the surface of epithelial cells in its native state. Contributions of adaptor proteins, membrane dynamics, and post-translational modifications that are present in the cell-based assay may therefore be unaccounted for in the pure system. Nonetheless, these data support the notion that J3F.1 and J10.4 bind to a region of JAM1 that may be implicated in homodimer formation and that this structural motif may be important for epithelial barrier function.

It is interesting that 1H2A9 was observed to block homophilic interactions at high concentrations but did not inhibit TER recovery. One possible explanation is that, unlike J3F.1/J10.4, which appear to directly disrupt the homodimer interface, 1H2A9 may be an “allostic” inhibitor that binds to a distant site and alters the conformation of JAM1. The lack of effect of 1H2A9 on TER recovery could therefore be explained by the presence of other proteins in cells that block the 1H2A9 epitope or stabilize JAM1 in a conformation that is made less susceptible to disruption by 1H2A9. Such stabilizing factors would not exist in the in vitro system used to test homophilic interactions.

Mutations within the Homodimer Interface Prevent JAM1 Enrichment at Junctions between Transfected Cells—It has been suggested that homophilic interaction between JAM1 molecules on adjacent cells stabilizes its localization at points of contact (1, 4). Here we have shown that deletion of the N-terminal Ig-like loop (mutant DL1) and mutations to specific residues within the putative homodimer interface (mutant E61R/K63E) dramatically alter this localization pattern. Unlike wild type JAM1, which we observed to localize exclusively at contacts between transfected cells (Fig. 8A), the DL1 and E61R/K63E mutants stained the entire circumference of cells, even in areas that did not touch other transfected cells (Fig. 8, C and D). The simplest explanation for this observation is that the homodimer interface mutants lack the adhesive properties required to stabilize JAM1 at points of cell contact, and thus they are free to diffuse throughout the cell membrane. These results could also be explained by more indirect mechanisms, such as failure of these mutants to activate signal transduction pathways critical for JAM1 localization to points of cell contact. In either case, the effects of the DL1 and E61R/K63E mutations suggest that the homodimer interface is important for localization of JAM1 and support our antibody-based experiments suggesting homodimer formation is critical for JAM1 function.

Interestingly, the N117K mutation was shown to block J3F.1 and J10.4 binding to JAM1 (Fig. 5) but did not affect localization of JAM1 at cell junctions (Fig. 8B). This result, however, is consistent with the model presented in the JAM1 crystal structure (17). As shown in Fig. 7A, residue Asn-117 is located on the turn between β-strands F and G, just adjacent to the homodimer interface, and is not predicted to be directly involved in homophilic interactions.

In addition to transfecting CHO cells with homodimer interface mutants, we transfected SKCO15 cells to investigate whether these mutations affected TER recovery. Interestingly,
we did not observe an effect on TER recovery with any mutant. We also tested peptides from Table I, but none of these peptides affected TER recovery either. We reason that the homoepitopic interactions of endogenous JAM1 are highly stable and not easily disrupted by small peptides or constructs containing mutations in the homoepitopic interface.

The Role of JAM1 Homomorphic Interaction in TJ—Previous studies (4) have shown that JAM1 forms homodimers in solution and on the surface of CHO cells transfected with exogenous JAM1. ELISAs and other binding assays have demonstrated that recombinant soluble extracellular JAM1 binds to immobilized JAM1 in microtiter wells (4). Studies of the crystal structures of murine and human JAM1 demonstrate the existence of homodimers under crystallization conditions (16, 17). Here we report identification of functional epitopes within JAM1 that overlap the putative homoepitopic interface. We have shown that mAbs J3F.1 and J10.4, which inhibit barrier recovery in epithelial cells, bind to a region of JAM1 implicated in homodimer formation and that J10.4 is capable of blocking JAM1 homodimer formation in vitro. In addition, we show that mutations within the JAM1 homoepitopic interface prevent enrichment of JAM1 at points of cell contact. Together, these results support the conclusion that homoepitopic JAM1 interactions are important for its function in epithelial cells.

Studies have also suggested that JAM1 participates in heterotypic interactions, namely with integrins (6, 14, 24). Similarly, JAM-2 and JAM-3 have been shown to interact with each other and with integrins simultaneously (25). The significance of heterotypic JAM1 interactions has primarily been studied within the context of leukocyte migration (6, 11, 14, 25) and angiogenesis (24). The role of heterotypic JAM1 interactions has not been characterized completely. Although we propose that homotypic JAM1 interactions are critical for epithelial barrier function, the contribution of heterotypic interactions to this process cannot be excluded. Antibodies J3F.1 and J10.4 appear to bind to a region of JAM1 implicated in homodimer formation, but it also possible that their effects on barrier recovery may be attributable to disruption of heterotypic interactions. Future studies that explore the structural basis for JAM1 function in epithelial cells should consider the potential contribution of both homotypic and heterotypic interactions.

In interpreting our epitope mapping results, it is important to consider other studies that have identified functional epitopes within the N-terminal Ig-like loop of JAM1. Bazzoni et al. (4), for example, have shown that the BV11 antibody, which was observed to inhibit leukocyte migration in endothelial cells (1) and block homodimer formation in vitro, may bind to residues 28–31 (4). Although this epitope appears to be distant from the J3F.1/J10.4 epitope in the primary structure of JAM1, analysis of the crystal structure suggests that these epitopes are actually in close spatial proximity, i.e. residues 28–31 reside on a β-strand just adjacent to residues 117–121 of the J3F.1/J10.4 epitope (not shown). Similarly, peptide-based epitope mapping experiments by Babinaka et al. (26) suggest that residues 28–50 are important for platelet aggregation. It is not clear, however, whether the functional epitopes described by Bazzoni (4) and Babinaka (26) are structurally and functionally distinct from the J3F.1/J10.4 epitope or whether these epitopes are part of a larger region that has functional significance in platelets and epithelial cells alike.

Acknowledgments—We thank Susan Voss for tissue culture expertise and Stephanie Burst for expert technical assistance.

REFERENCES
1. Martin-Padura, I., Lostaglio, S., Schneemann, M., Williams, L., Romano, M., Fruscella, P., Panzeri, C., Stoppani, A., Ruoč, L., Villa, A., Simmons, G., and Dejana, E. (1998) J. Cell Biol. 142, 117–127
2. Williams, L. A., Martin-Padura, I., Dejana, E., Hogg, N., and Simmons, D. L. (1999) Mol. Immunol. 36, 1175–1188
3. Liu, Y., Nusrat, A., Schmaier, A. H., Walsh, S., Pochet, M., and Parkos, C. A. (2000) J. Cell Sci. 113, 2363–2374
4. Bazzoni, G., Martinez-Estrada, O. M., Mueller, F., Nelboeck, P., Schmid, G., Bartfai, T., Dejana, E., and Brockhaus, M. (2000) J. Biol. Chem. 275, 30970–30976
5. Liang, T. W., DeMarco, R. A., Mrny, R. J., Gurney, A., Gray, A., Hooley, J., Aaron, H. L., Huang, A., Klassen, T., Tumas, D. B., and Fong, S. (2000) Am. J. Physiol. 279, C1733–C1743
6. Ostermann, G., Weber, K. S., Zernecke, A., Schroder, A., and Weber, C. (2002) Nat. Immunol. 3, 151–158
7. Del Maschio, A., De Luigi, A., Martin-Padura, I., Brockhaus, M., Bartfai, T., Fruscella, P., Adorini, L., Martino, G., Furlan, R., De Simoni, M. G., and Dejana, E. (1999) J. Exp. Med. 190, 1351–1356
8. Elnest, K., Schulz, C. U., Meyer Za Bruckwedde, M. K., Pendl, G. G., and Vestweber, D. (2000) J. Biol. Chem. 275, 27679–27688
9. Bazzoni, G., Martinez-Estrada, O. M., Orsenigo, F., Cordenonsi, M., Citi, S., and Dejana, E. (2000) J. Biol. Chem. 275, 20280–20286
10. Cunningham, S. A., Arrate, M. P., Rodriguez, J. M., Bjercke, R. J., Vanderslice, J. M., Arroyo, M. A., and Brock, T. A. (2000) J. Biol. Chem. 275, 34750–34756
11. Johnson-Leger, C. A., Aurand-Lions, M., Beltramelli, N., Pasel, N., and Imhof, B. A. (2002) Blood 99, 2749–2756
12. Arrate, M. P., Rodriguez, J. M., Tran, T. M., Brock, T. A., and Cunningham, S. A. (2001) J. Biol. Chem. 276, 45826–45832
13. Liang, T. W., Chiu, H. H., Gurney, A., Sidie, A., Tumas, D. B., Schow, P., Foster, J., Klassen, T., Dennis, K., DeMarco, R. A., Pham, T., Frantz, G., and Fong, S. (2002) J. Immunol. 168, 1618–1620
14. Santoso, S., Sachs, U. J., Krohl, H., Linder, M., Ruf, A., Preissner, K. T., and Chavakis, T. (2002) J. Biol. Chem. 277, 679–691
15. Hirabayashi, S., Tajima, M., Yeo, I., Nishimura, W., Mori, H., and Hata, Y. (2003) Mol. Cell. Biol. 23, 4267–4282
16. Kostrewa, D., Brockhaus, M., D’Arcy, A., Dale, G. E., Nelboeck, P., Schmid, G., Muehler, F., Bazzoni, G., Dejana, E., Bartfai, T., Winkler, T., Kuhn, F. K., and Hennig, M. (2001) EMBO J. 20, 4391–4398
17. Prota, A. E., Campbell, J. A., Schelling, P., Forrest, J. C., Watson, M. J., Peters, T. R., Aurand-Lions, M., Imhof, B. A., Demody, T. S., and Stehle, T. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 5366–5371
18. Naik, U. P., Ehrlich, Y. H., and Kornecki, E. (1995) J. Biol. Chem. 270, 16974–16980
19. Lisanti, M. P., Caras, I. W., Davitz, M. A., and Rodriguez-Boulan, E. (1989) J. Cell Biol. 109, 2175–2185
20. Babinska, A., Kedees, M. H., Athar, H., Sobocka, T., Sobocka, M. B., Ahmed, T., Ehrlich, Y. H., Hussain, M., and Korczeni, E. (2002) Thromb. Haemostasis 87, 712–721
Involvement of the Junctional Adhesion Molecule-1 (JAM1) Homodimer Interface in Regulation of Epithelial Barrier Function
Kenneth J. Mandell, Ingrid C. McCall and Charles A. Parkos

J. Biol. Chem. 2004, 279:16254-16262.
doi: 10.1074/jbc.M309483200 originally published online January 28, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309483200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 26 references, 20 of which can be accessed free at http://www.jbc.org/content/279/16/16254.full.html#ref-list-1