Integrin α4β1-Dependent Adhesion to ADAM 28 (MDC-L) Requires an Extended Surface of the Disintegrin Domain†

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ABSTRACT: ADAMs (a disintegrin and metalloprotease) are a family of proteins that possess functional adhesive and proteolytic domains. ADAM 28 (MDC-L) is expressed by human lymphocytes and contains a disintegrin-like domain that serves as a ligand for the leukocyte integrin, α4β1. To elucidate which residues comprise the α4β1 binding site in the ADAM 28 disintegrin domain, a charge-to-alanine mutagenesis strategy was utilized. Each alanine substitution mutant was evaluated and compared to the native sequence for its ability to support cell adhesion of the T-lymphoma cell line, Jurkat. This approach identified ADAM 28 residues Lys^{437}, Lys^{442}, Lys^{455}, Lys^{459}, Lys^{460}, Lys^{469}, and Glu^{476} as being essential for α4β1-dependent cell adhesion. The epitope for a function-blocking monoclonal antibody, Dis 1-1, was localized to the N-terminal end of the ADAM 28 disintegrin domain using these same charge-to-alanine mutants. Three distinct molecular models based upon the known structures of snake venom disintegrins suggested that residues contributing to α4β1 recognition are aligned on one face of the domain. This study demonstrates that residues located outside of the disintegrin loop participate in integrin recognition of mammalian disintegrins.

The ADAM[1] (a disintegrin and metalloprotease) family of proteins is comprised of more than 30 transmembrane and secreted glycoproteins that are capable of both proteolytic and adhesive functions (*1*, *2*). ADAMs are typically comprised of a prodomain and metalloprotease, disintegrin-like, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane, and cytoplasmic domains (*3*). The role of ADAMs in the cell surface processing of several important molecules such as tumor necrosis factor-α by ADAM 17 (TACE) (*4*, *5*), the proper cleavage of the amyloid precursor protein by ADAM 10 and 17 (*6*, *7*), and shedding of membrane-anchored heparin-binding EGF-like growth factor by ADAM 9 (*8*) has been clearly demonstrated. Recently, members of a new family of proteins closely related to the ADAM protein family, ADAMTS (a disintegrin and metalloprotease with thrombospondin motifs), have been shown to exhibit proteolytic activity toward numerous matrix constituents such as aggrecan, procollagen, versican, and brevican (*9*−*13*). This suggests that the proteolytic role of the ADAMs may not be restricted to ectodomain shedding events, but may also involve the processing of the extracellular matrix.

Another functional characteristic of the ADAM family is their potential to serve as integrin ligands, a premise initially based upon the extensive homology of the ADAM disintegrin-like domains with small nonenzymatic peptides found in the venom of snakes. Snake venom disintegrins are integrin antagonists that disrupt normal platelet aggregation (*14*, *15*). The ability of snake venom disintegrins to act as integrin antagonists has been attributed to an extended loop within the snake venom disintegrin structure where an Arg-Gly-Asp (RGD) or Lys-Gly-Asp (KGD) integrin recognition sequence is located (*16*−*18*). Several ADAMs have been demonstrated to mediate adhesive events by interacting with members of the integrin family. ADAM 2, 3, and 9 are recognized by α6β1 (*19*−*22*) with ADAM 9 also being recognized by αvβ5 (*23*). ADAM 12 and 15 are recognized by α9β1 (*24*) with ADAM 15 also being recognized by αvβ3 and α5β1 (*25*, *26*). ADAM 23 is recognized by αvβ3 (*27*), and ADAM 28 is recognized by the leukocyte integrin α4β1 (*28*).

Even though ADAMs are well-established as integrin ligands, the exact nature of this recognition and how mammalian disintegrins function as integrin ligands are poorly understood. The "disintegrin loop", a segment within the ADAM disintegrins corresponding to the location of the RGD sequence in the snake venom disintegrins, has been demonstrated to contain residues that are critical for integrin recognition of ADAM 2, 3, 15, and 23 (*19*, *21*, *24*, *27*, *29*, *30*). This suggests a conserved mechanism of integrin recognition between mammalian and snake venom disinte-

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[1] Abbreviations: ADAM, a disintegrin and metalloprotease; BSA, bovine serum albumin; CS-1, connecting segment-1 of fibronectin; EGF, epidermal growth factor; ELISA, enzyme-linked immunosorbent assay; KGD, Lys-Gly-Asp; mAb, monoclonal antibody; NMR, nuclear magnetic resonance; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; RGD, Arg-Gly-Asp; VCAM-1, vascular cell adhesion molecule-1; SD, standard deviation.

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grins. However, recent mutational studies suggest that residues outside of the disintegrin loop of mammalian disintegrins are critical for integrin-dependent adhesion (*24*, *31*, *32*). The larger mammalian disintegrins may utilize additional regions of the domain when compared to the smaller snake venom disintegrins.

We have recently demonstrated that ADAM 28 (MDC-L) interacts with the leukocyte integrin α4β1 in an activation-dependent manner (*28*). ADAM 28 exhibits a prototypical ADAM domain structure, containing an active metalloprotease (*33*, *34*). Human ADAM 28 expression appears to be lymphocyte specific and has been identified as a potential cellular marker for B-cell chronic lymphocytic leukemia by gene expression profiling (*35*, *36*). ADAM 28 lacks an IELDVPST or QIDSP sequence that facilitates α4β1-dependent recognition of the alternatively spliced connecting segment of fibronectin (CS-1) or vascular cell adhesion molecule-1 (VCAM-1), respectively (*37*−*40*). Therefore, the molecular mechanism of recognition appears to be distinct from the mechanism of these well-characterized α4β1 ligands. Here we identify multiple charged residues within the disintegrin domain of ADAM 28 that are essential for α4β1-dependent cell adhesion. In addition, the epitope for a function-blocking monoclonal antibody (mAb), Dis 1-1 (*28*), is localized. Modeling studies that are presented suggest that residues critical for integrin recognition are localized to a particular face of the ADAM 28 disintegrin domain, not to a single loop.

EXPERIMENTAL PROCEDURES

Materials. The Jurkat cell line was purchased from the American Type Culture Collection (Manassas, VA). Jurkat cells were maintained in RPMI 1640 containing 10 mM HEPES, 1 mM sodium pyruvate, 10% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. The QE2E5-producing hybridoma cell line (*41*) was generously provided by R. Faull (Royal Adelaide Hospital, Adelaide, Australia). The mAb Dis 1-1 has been previously described (*28*). The function-blocking anti-α4 mAb P1H4 was purchased from Chemicon International, Inc. (Temecula, CA). The CS-1 peptide (EILDVPST) was obtained from the American Peptide Company, Inc. (Sunnyvale, CA).

rDis−*Fc Fusion Proteins.* Recombinant DNA methods for the generation of the rDis−Fc construct were previously described (*28*). The Insect Select vector pIB/V5-His (Invitrogen, Carlsbad, CA) was utilized for expression of all rDis−Fc fusion proteins. Briefly, cDNA encoding ADAM 28 residues T^{407}−H^{664} with unique *Xba*I and *Bgl*II sites on the ends was generated by PCR, and the product was cloned in frame with the GP67 insect signal sequence of the vector pAc-GP67 (BD PharMingen, San Diego, CA). A PCR product spanning the GP67-ADAM 28 disintegrin domain (residues T^{407}−G^{500}) was then fused in frame to the amino terminus of the Fc region of a human IgG_3 heavy chain lacking the hinge region and containing a carboxyl-terminal stop codon by PCR overlap extension. The rDis−Fc construct was subsequently subcloned into the TOPO pIB/V5-His vector. After verification by nucleotide sequencing, the DNA construct was transfected into High 5 cells with Insect Plus Liposome (Invitrogen). Selection was performed with medium containing blasticidin, until stable transfected cell lines were generated.

Alanine substitution mutants were generated with the Quick Change site-directed mutagenesis kit (Stratagene, La Jolla, CA). Oligonucleotides were synthesized by the Molecular Biology Resource Facility at The University of Oklahoma Health Sciences Center. Each mutant was verified by complete nucleotide sequencing of the ADAM 28 disintegrin domain coding region before transfection of the plasmid into High 5 cells. Purified recombinant protein was obtained by applying polyethylene glycol concentrated conditioned medium to protein-G Sepharose (Pharmacia, Uppsala, Sweden). The protein was eluted from the protein-G Sepharose by application of 100 mM citric acid (pH 3.0) followed by immediate neutralization of the fractions by addition of 1 M Tris-HCl (pH 9.0).

Cell Adhesion Assay. The assay that was used was adapted from that previously described (*42*). Purified Fc fusion proteins were coated on Immulon-2 96-well plates (Dynatech Laboratories, Inc., Chantilly, VA) at various concentrations in 0.1 M $NaHCO_3$ (pH 8.4) overnight at 4 °C in a total volume of 100 μL. The protein was decanted, and the wells were subsequently blocked with 4% bovine serum albumin (BSA) in 0.1 M $NaHCO_3$ (pH 8.4) at room temperature for 2 h. Jurkat cells were washed three time with HEPES Tyrode's buffer (5 mM HEPES, 150 mM NaCl, 12 mM $NaHCO_3$, 2.6 mM KCl, 5 mM D-glucose, 0.2 mg/mL BSA, 0.5 mM $MgCl_2$, and 1 mM $CaCl_2$). Before addition to wells, the cells were resuspended to a concentration of 2×10^6 cells/mL in HEPES Tyrode's buffer containing 10 μg/mL anti-β1 activating mAb QE2E5. Cells (100 μL) were immediately added to washed wells and incubated with immobilized substrates for 2 h at 37 °C in an atmosphere of 5% CO_2. Control experiments included the α4β1 inhibitor mAb P1H4 (10 μg/mL) or CS-1 peptide (200 μg/mL). The wells were gently washed three times with HEPES Tyrode's buffer to remove nonadherent cells. Adherent cells in each well were detected by colormetric determination of endogenous cellular acid phosphotase activity by incubation in 100 μL of 1% Triton X-100, 50 mM sodium acetate (pH 5.0), and 6 mg/mL *p*-nitrophenyl phosphate at 37 °C for 1 h. After the wells had been developed by the addition of 50 μL/well of 1 N NaOH, the plates were read at 405 nm. Quantification of the number of adherent cells was based upon a standard curve generated from a known number of Jurkat cells for each experiment.

Epitope Mapping of mAb Dis 1-1. Enzyme-linked immunosorbent assays (ELISA) were based on standard procedures (*43*). Purified Fc fusion proteins were diluted to 5 μg/mL in 0.1 M $NaHCO_3$ (pH 8.4); 50 μL/well was added to Immulon-2 96-well plates, and the plates were incubated overnight at 4 °C. Wells were decanted and incubated at room temperature for 1 h with 2% blotto in phosphate-buffered saline (PBS) to prevent nonspecific binding. After three washes with PBS containing 0.05% Tween 20, 1 μg/mL mAb Dis 1-1 was added (50 μL/well) and incubated for 2 h at room temperature. The plates were washed again, and the secondary goat anti-mouse biotin-conjugated antibody (Pierce, Rockford, IL) diluted 1:5000 in PBS was added. Incubation with the secondary antibody was performed at room temperature for 1 h. Washes were repeated, and bound biotinylated antibody was detected with avidin and biotinylated horseradish peroxidase (Vector Laboratories, Inc., Burlingame, CA). The assays were developed using *o*-

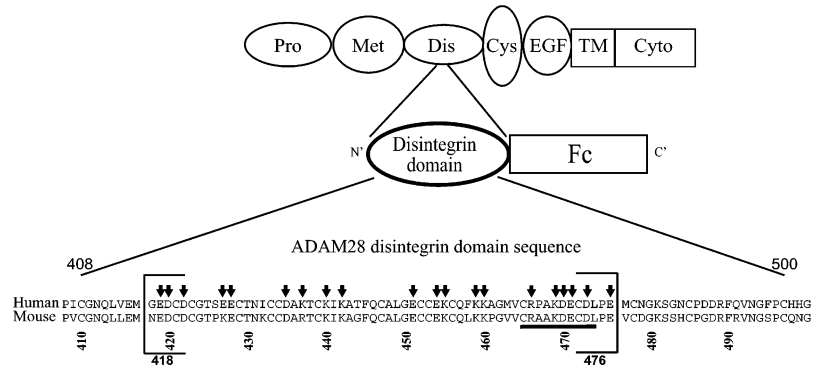

FIGURE 1: Charge-to-alanine mutagenesis of the ADAM 28 disintegrin domain. The full-length ADAM 28 domain structure is depicted at the top. The diagram of the recombinant disintegrin Fc fusion protein (rDis−Fc) secreted from stable transfected insect cells utilized in the adhesion and epitope localization experiments is featured. A primary sequence comparison of the disintegrin domains from human ADAM 28 and the mouse homologue is shown. Arrows indicate charged residues targeted for alanine substitution. Brackets enclose the 59-residue region of human ADAM 28 sufficient for cell adhesion with the residues comprising the putative disintegrin loop denoted with a bold underscore.

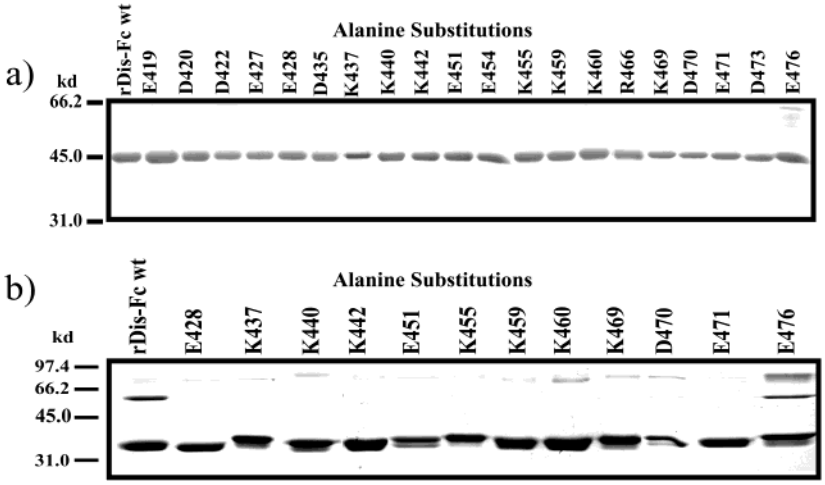

FIGURE 2: SDS−PAGE analysis of purified recombinant native and alanine-substituted ADAM 28 disintegrin domains. SDS−PAGE (10%) analysis of purified rDis−Fc proteins eluted from protein-G Sepharose and used in adhesion experiments. The proteins were run under reducing (a) or nonreducing (b) conditions at 3 or 5 μg/lane, respectively. Only alanine replacements that adversely effected $\alpha 4\beta 1$-dependent cell adhesion to the rDis−Fc fusion protein were analyzed under nonreducing conditions. Total protein was detected with Coomassie blue. Molecular mass standards are shown at the left.

phenylenediamine, and absorbance measurements were collected at 490 nm.

Molecular Models. The molecular models of the ADAM 28 disintegrin domain were constructed by homology modeling using three distinct snake venom disintegrin NMR structures. The NMR coordinates for echistatin (PDB entry 2ECH), flavoridin (PDB entry 1FVL), and kistrin (PDB entry 1KST) were obtained from the Protein Data Bank (*44*). Residue replacement was performed for each template utilizing Main 99 (*45*) software on a Silicon Graphics workstation. Insertion of unique ADAM 28 residues was done after completion of residue replacement. Subsequent minimization of models was achieved with Insight II (Biosym Technologies, San Diego, CA) after soaking each model in five shells of minimized water. A set of core residues was not evident due to the limited size of the models. Consequently, all residues were minimized simultaneously without constraint. Steepest-descent minimizations were conducted in four consecutive steps at 500 iterations per step. Initially, all main chain hydrogen atoms were minimized with subsequent minimizations adding in succession the main chain heavy atoms, side chain hydrogens, and side chain heavy atoms. A minimization step using the conjugate gradient algorithm at 1000 iterations was then applied to the models. Upon completion, all models were visually inspected for alignment of side chains and disulfide bridge formation.

RESULTS

Identification of ADAM 28 Residues Required for $\alpha 4\beta 1$-Dependent Jurkat Cell Adhesion. We have previously demonstrated that ADAM 28 is a ligand for the leukocyte integrin $\alpha 4\beta 1$ (*28*). In this study, a charge-to-alanine mutagenesis strategy was employed to identify which residues in the ADAM 28 disintegrin domain contribute to $\alpha 4\beta 1$-dependent Jurkat cell adhesion. Charged residues conserved between the human and mouse homologues were targeted for alanine substitution by site-directed mutagenesis (Figure 1). The ADAM 28 disintegrin domains possessing either the native sequence or the designated alanine substitution were recombinantly expressed as human Fc fusion proteins (rDis−Fc) by stable transfection of insect cells with subsequent purification of the secreted recombinant proteins from the conditioned medium. Via SDS−PAGE analysis, each protein-G Sepharose-purified rDis−Fc alanine substitution mutant exhibited a similar migration pattern and purity

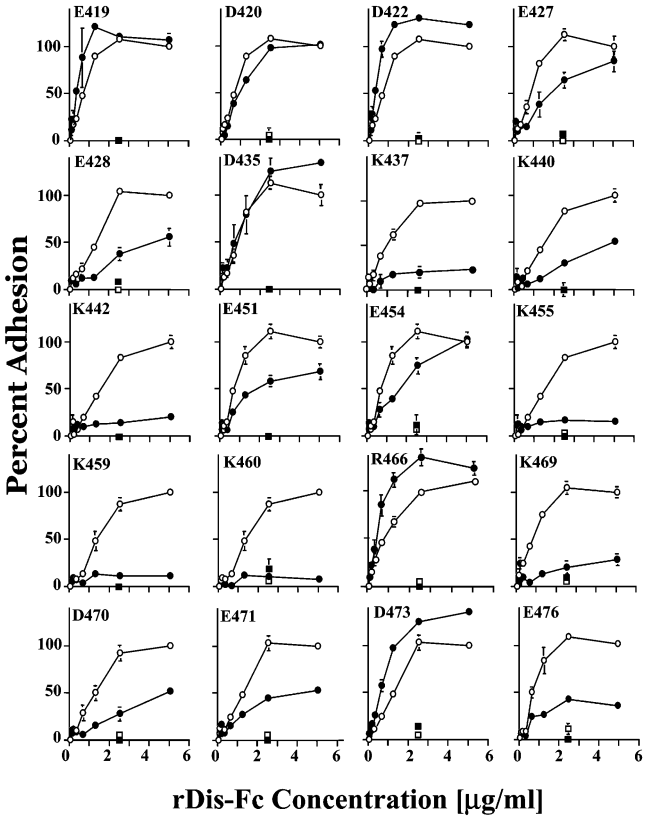

FIGURE 3: Residues required for α4β1-dependent Jurkat cell adhesion to the disintegrin domain of ADAM 28. Microtiter wells were coated with varying concentrations of either the native rDis−Fc protein or the indicated charge-to-alanine mutant. Jurkat cells (2×10^5 cells/well) were added to wells in modified Tyrode's buffer containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 10 mg/mL mAb QE2E5 to ensure exogenous activation of the β1 integrins. The relative number of adherent cells was determined by quantifying the endogenous acid phosphatase activity and comparing experimental values to a standard curve (see Experimental Procedures). The relative ability of the native rDis−Fc protein (○) and charge-to-alanine-substituted rDis−Fc proteins (●) to support Jurkat cell adhesion is shown. Inhibition of α4β1-dependent adhesion was performed for both native (□) and charge-to-alanine-substituted (■) rDis−Fc proteins (2.5 μg/mL) in the presence of 10 μg/mL mAb P1H4. Results are averages ± standard deviation from a representative experiment performed in triplicate. Percent adhesion = [adherent $cells_{(mutant\ rDis-Fc)}$ − adherent $cells_{(BSA)}$]/[adherent $cells_{(native\ rrDis-Fc\ at\ 5\ \mu g/mL)}$ − adherent $cells_{(BSA)}$] × 100.

when compared to the native rDis−Fc fusion protein (Figure 2). This suggested that the purified recombinant proteins contained no gross structural perturbations resulting in aberrant disulfide bond formation.

The purified charge-to-alanine mutants were then assessed for their relative ability to support Jurkat cell adhesion when compared to the native disintegrin domain (Figure 3). Results shown in Figure 3 are from a single representative experiment. The average concentration required for each charge-to-alanine substitution to achieve 50% of the adhesion obtained with the native rDis−Fc fusion protein at 5 μg/mL was determined from three independent experiments, and the results are shown in Table 1. Alanine substitutions of residues Lys^{437}, Lys^{442}, Lys^{455}, Lys^{459}, Lys^{460}, Lys^{469}, and Glu^{476} substantially affected the ability of the rDis−Fc fusion protein to support Jurkat cell adhesion. Substitution at residues Glu^{428}, Lys^{440}, Glu^{451}, Asp^{470}, and Glu^{471} also affected Jurkat cell adhesion to the recombinant ADAM 28 disintegrin domain, requiring 2−5-fold higher concentrations than the native rDis−Fc fusion protein to achieve the same level of adhesion. Jurkat cell adhesion to the rDis−Fc alanine substitutions and the native sequence was shown to be α4β1-dependent as residual activity was inhibited by the anti-α4 mAb P1H4 (Figure 3) and the CS-1 peptide (data not shown). In support of the adhesion results, fluorescein isothiocyanate labeling of the native rDis−Fc fusion protein via primary amines abolished solution binding of this ligand to α4β1 on the surface of Jurkat cells, suggesting that lysine residues of ADAM 28 participate in α4β1 interactions (data not shown). Interestingly, the majority of the residues shown to be involved in α4β1 recognition are not contained in the putative disintegrin loop sequence of Asp^{470}−Asp^{473} that corresponds to the RGD location within the snake venom disintegrins. In addition, alanine substitution of Arg^{466} and Asp^{473}, residues conserved in many ADAMs previously shown to be essential for α6β1− and α9β1−disintegrin interactions, had no effect on cell adhesion to the ADAM 28 disintegrin domain (*19*, *29*, *31*). These data demonstrate that α4β1-dependent recognition of the ADAM 28 disintegrin domain requires multiple charged residues that are linearly distant from the putative disintegrin loop, a region previously implicated in adhesion of other disintegrins.

Table 1: Concentration of the rDis−Fc Fusion Protein Required To Achieve 50% Adhesion of the Native rDis−Fc Protein

residue being mutated	50% native adhesion[a] ± SD (μg/mL)
native	1.00 ± 0.21
E^{419}	0.44 ± 0.10
D^{420}	0.98 ± 0.40
D^{422}	0.49 ± 0.16
E^{427}	1.40 ± 0.57
E^{428}	2.21 ± 1.30
D^{435}	0.87 ± 0.35
K^{437}	**>5**
K^{440}	4.67 ± 0.58
K^{442}	**>5**
E^{451}	2.78 ± 1.93
E^{454}	1.56 ± 0.89
K^{455}	**>5**
K^{459}	**>5**
K^{460}	**>5**
R^{466}	0.52 ± 0.12
K^{469}	**>5**
D^{470}	4.68 ± 0.30
E^{471}	2.74 ± 0.19
D^{473}	0.76 ± 0.10
E^{476}	**>5**

[a] The 50% adhesion values are the concentrations of ligand required to reach 50% of cell adhesion that was achieved with 5 μg/mL native rDis−Fc protein. The 50% adhesion values and standard deviations for alanine mutants were obtained from three separate experiments. Native rDis−Fc values were obtained from 10 separate experiments.

Localization of the Epitope for the Inhibitory mAb Dis 1-1. The mAb Dis 1-1 inhibits α4β1-dependent cell adhesion to the immobilized rDis−Fc fusion protein (*28*). The epitope for this mAb was localized to a 59-residue segment (Gly^{418}−Glu^{476}) of the ADAM 28 disintegrin domain that was also sufficient for promoting cell adhesion. The same charge-to-alanine mutants shown in Figure 1 were utilized in an ELISA to localize the mAb Dis 1-1 epitope (Figure 4). Three charged residues (Glu^{419}, Asp^{420}, and Asp^{422}) positioned at the N-terminus of the Gly^{418}−Glu^{476} region significantly affected

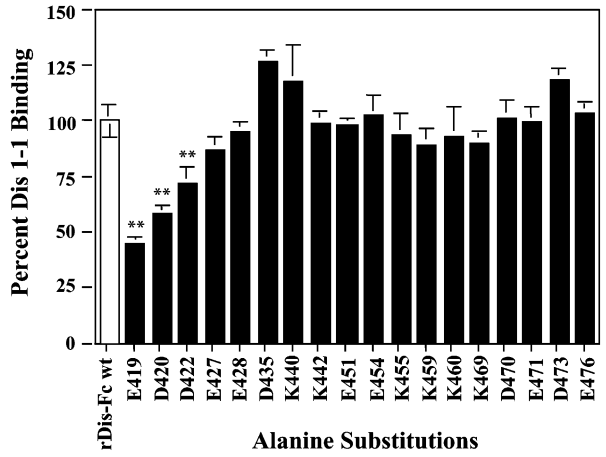

FIGURE 4: Epitope for the inhibitory mAb Dis 1-1 localized to residues E^{419}, D^{420}, and D^{422} of ADAM 28. Microtiter wells were coated with 5 μg/mL native or mutant rDis−Fc protein. After blocking had been carried out, the wells were incubated with 1 μg/mL mAb Dis 1-1. Bound mAb Dis 1-1 was detected with peroxidase-conjugated goat anti-mouse IgG specific antibody and developed with *o*-phenylenediamine. Percent mAb Dis 1-1 binding $= [A_{490(\text{mutant rDis−Fc})} - A_{490(\text{blotto})}]/[A_{490(\text{native rDis−Fc})} - A_{490(\text{blotto})}] \times 100$. Results shown for the native disintegrin domain (white bar) were the result of 10 separate determinations conducted in triplicate (average ± SD). Data displayed for charge-to-alanine mutants (black bars) represent three separate experiments each performed in triplicate (average ± SD). Two asterisks indicate that a Student's *t* test resulted in a *p* of <0.01 relative to mAb Dis 1-1 recognition of the native rDis−Fc protein.

mAb Dis 1-1 recognition of the rDis−Fc fusion protein when individually substituted with alanine. These same three alanine substitutions had no significant effect on Jurkat cell adhesion (Table 1). The epitope for the mAb Dis 1-1 and the residues required for $\alpha4\beta1$-dependent adhesion appeared to be distinct; however, the mAb Dis 1-1 epitope may be adjacent to the integrin recognition site so that the mAb could sterically inhibit the $\alpha4\beta1$−disintegrin domain interaction. To better evaluate the results obtained with our charge-to-alanine analysis, molecular modeling studies of the ADAM 28 disintegrin domain were initiated.

Molecular Modeling of the ADAM 28 Disintegrin Domain. Since there are no mammalian disintegrin domain structures available, we used the NMR-derived structures from three snake venom disintegrins that included echistatin, flavoridin, and kistrin for modeling templates (*16*, *18*). Primary sequence alignment of echistatin, flavoridin, and kistrin with the ADAM 28 disintegrin domain sequence is shown in Figure 5a. The placement of the template sequence with respect to the ADAM 28 sequence corresponds to the region of ADAM 28 modeled for that template. Even though the disulfide bond pattern was nearly identical for each of the three templates, with the cysteine pairing between the flavoridin and kistrin structures being completely conserved, each was structurally distinct (Figure 5b) (*46*). Because of these structural differences, three separate structural models of the ADAM 28 disintegrin domain were generated (Figure 6).

Evaluation of the three models revealed that alanine substitutions which effect adhesion of Jurkat cells to the rDis−Fc fusion protein are dispersed on the surface of each model and not exclusively localized to the putative disintegrin loop. Collectively, these three models suggested that the ADAM 28 disintegrin domain supports $\alpha4\beta1$-dependent adhesion via an extended surface of the domain. The two models based upon echistatin and flavoridin appear to be less globular than that generated from kistrin. The molecular models based on echistatin and flavoridin suggested that residues critical for integrin-dependent adhesion are primarily located on the same face of the molecule. Furthermore, the epitope for the function-blocking mAb Dis 1-1 on these models was localized at the end of the molecule most distant from the disintegrin loop, further suggesting that $\alpha4\beta1$ recognition of the ADAM 28 disintegrin domain requires residues outside the disintegrin loop.

DISCUSSION

In this study, we identified charged residues within the ADAM 28 disintegrin domain that function in $\alpha4\beta1$-dependent cell adhesion. Molecular models based upon three snake venom disintegrin structures were generated to visualize the localization of residues comprising the $\alpha4\beta1$ integrin recognition site and the epitope of the mAb Dis 1-1 within the ADAM 28 disintegrin domain. The results presented here indicate that integrin recognition of a mammalian disintegrin requires an extended surface comprised of multiple charged residues, a mechanism potentially used by all mammalian disintegrins for generating integrin recognition. Furthermore, this study confirms that residues outside the disintegrin loop are essential for integrin recognition of a mammalian disintegrin.

The role of snake venom disintegrins as integrin antagonists has been attributed to a region that displays an RGD or KGD integrin recognition sequence on an extended loop (*16*−*18*). Even though only one ADAM family member possesses an RGD sequence, ADAM 15, extensive homology exists between the disintegrin domains of the ADAMs and snake venom disintegrins (*15*). A region of the ADAM disintegrin domain known as the disintegrin loop that corresponds to the RGD location within the snake venom disintegrins has been implicated in integrin-mediated adhesion to several ADAMs, including ADAM 2, 3, 15, and 23 (*19*, *27*, *29*−*31*). Mutational and peptide analysis of integrin−disintegrin interaction has been predominately limited to this region of ADAM disintegrin domains. Results presented here support the contribution of additional regions of the ADAM 28 disintegrin domain in integrin-dependent adhesion. On the basis of the primary sequence (Figure 1) and the molecular models (Figure 6), most of these residues are not included in the putative disintegrin loop of ADAM 28. Although alanine substitutions of Asp^{470}, Glu^{471}, and Lys^{469} all adversely affected adhesion, Lys^{469} was the only residue contained within the putative loop that, when mutated to alanine, substantially altered the adhesive properties of the rDis−Fc fusion protein. These findings demonstrate that the adhesive component of the ADAM 28 disintegrin domain and potentially other mammalian ADAMs is not completely contained within the putative disintegrin loop region. Instead, integrin interaction with the ADAM 28 disintegrin relies upon multiple charged contacts that appear to be presented on an extended surface of the entire domain.

Since several ADAM disintegrins have been shown to act as integrin ligands, it is plausible that a set of residues conserved among the ADAMs create a general integrin recognition motif, whereas other residues unique to each

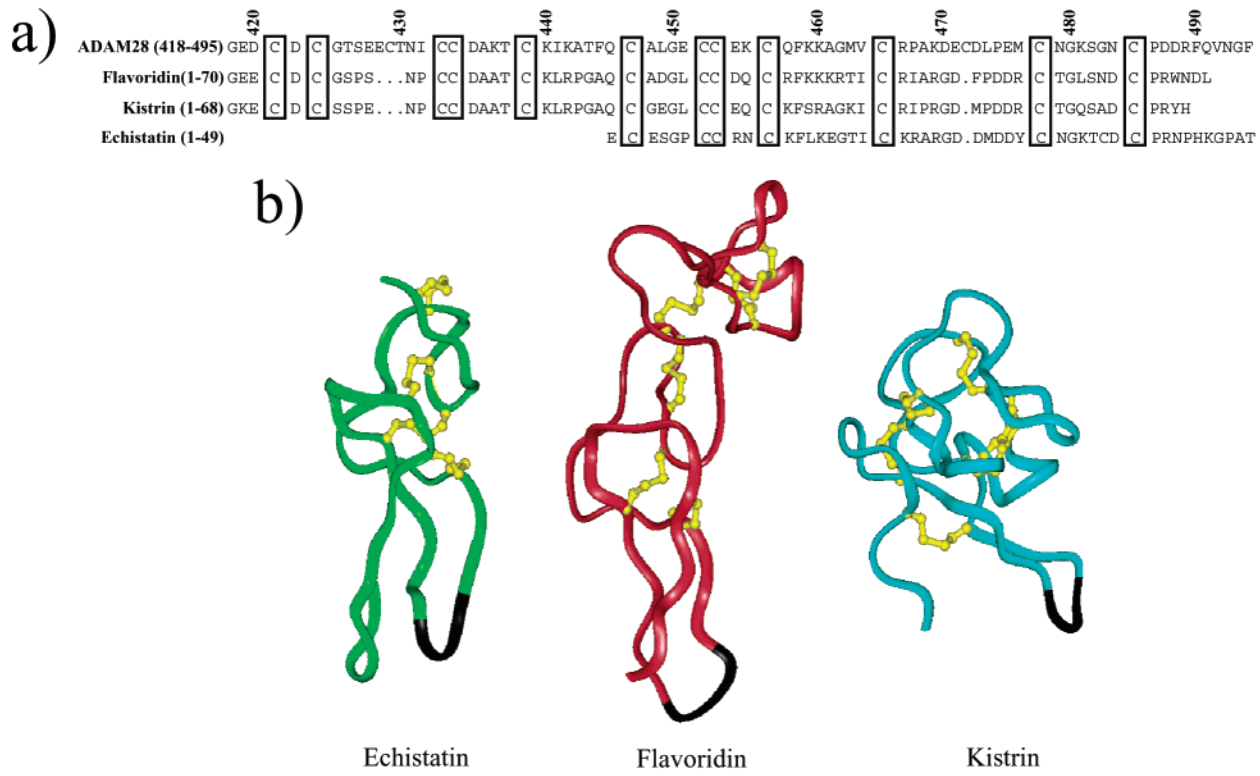

FIGURE 5: Templates employed in structural modeling of the ADAM 28 disintegrin domain. (a) Primary sequence alignment of the ADAM 28 disintegrin domain and the three snake venom disintegrins utilized as templates. Conserved cysteines in all four sequences are boxed. The residue number is with respect to ADAM 28 (*35*). (b) Ribbon diagram of the snake venom disintegrins echistatin (green), flavoridin (red), and kistrin (blue). The disulfides are depicted in yellow, and the position of the RGD-containing disintegrin loop is black.

ADAM may govern integrin specificity. A recent study by Eto et al. (*31*) examined the residues responsible for α9β1 recognition of ADAM 15. Thorough site-directed mutagenesis of the putative disintegrin loop of ADAM 15 established that a single motif composed of R(X6)DLPEF is essential for ADAM 15 binding to α9β1. Since this motif was conserved in 28 of the 30 ADAMs that were examined, it may function as a general recognition motif, but not be sufficient for imparting integrin specificity. Swapping of specific residues between the snake venom disintegrins echistatin and eristostatin demonstrated that specific residues dictate integrin specificity (*47*). Additionally, evidence also demonstrated that specific conserved residues are part of a general integrin recognition motif (*47*). The study presented here also supports the premise that a general integrin recognition motif exists for mammalian disintegrins. For example, Glu^{476} within the ADAM 28 sequence was shown to be absolutely critical in supporting Jurkat cell adhesion, and Glu^{491}, the corresponding residue in ADAM 15, was shown to be essential in mediating cell adhesion via α9β1 (*31*). This oxygenated residue may play a role similar to that of the Asp residue in the classical Arg-Gly-Asp integrin recognition sequence. However, it appears that other residues may determine the ability of an integrin to discriminate between the various ADAM disintegrins. Arg^{481} and Asp^{488} in ADAM 15 were also shown to be necessary for the adhesive properties of this disintegrin domain. These residues are conserved in multiple ADAMs, including ADAM 2 and ADAM 3, and have also been shown to be integral in the adhesive property of these two mammalian disintegrins (*19*, *29*). When the corresponding Arg^{466} and Asp^{473} residues were replaced with alanines in the ADAM 28 disintegrin domain, no detectable alteration in the ability of the ADAM 28 rDis−Fc protein to support α4β1-dependent cell adhesion was observed (Figure 3 and Table 1). These substitutions, as with all the alanine substitutions that retained adhesive activity, supported Jurkat cell adhesion via the integrin α4β1 as mAbs and the CS-1 peptide inhibited this adhesion. It should also be noted that the recombinant disintegrin domains expressed in our study were produced as secreted proteins from eukaryotic cells and not as refolded prokaryotic proteins as in other studies. Since the bacterially expressed ADAM 28 disintegrin domain supported α4β1-dependent adhesion (*28*), the differences in the production methods are not likely to account for the differences observed in residue requirements for integrin recognition.

The three distinct structural models presented reveal that the residues involved in α4β1 integrin-dependent cell adhesion to the ADAM 28 disintegrin domain are not exclusively located in the disintegrin loop. In particular, lysine residues spatially located throughout the domain appear to have an essential role in the ADAM 28−α4β1 interaction. Interestingly, all of the residues shown to be critical upon alanine substitution were distinctly localized to only one face of the echistatin-based model surface. The kistrin-based model exhibits a less confined arrangement of critical residues. More detailed structural analyses are needed for definitive localization of the residues that comprise the integrin recognition site of ADAM 28.

We previously evaluated alanine substitutions at Glu^{471} and Asp^{473} for their effect on Jurkat adhesion to the ADAM 28 rDis−Fc recombinant protein due to their importance in

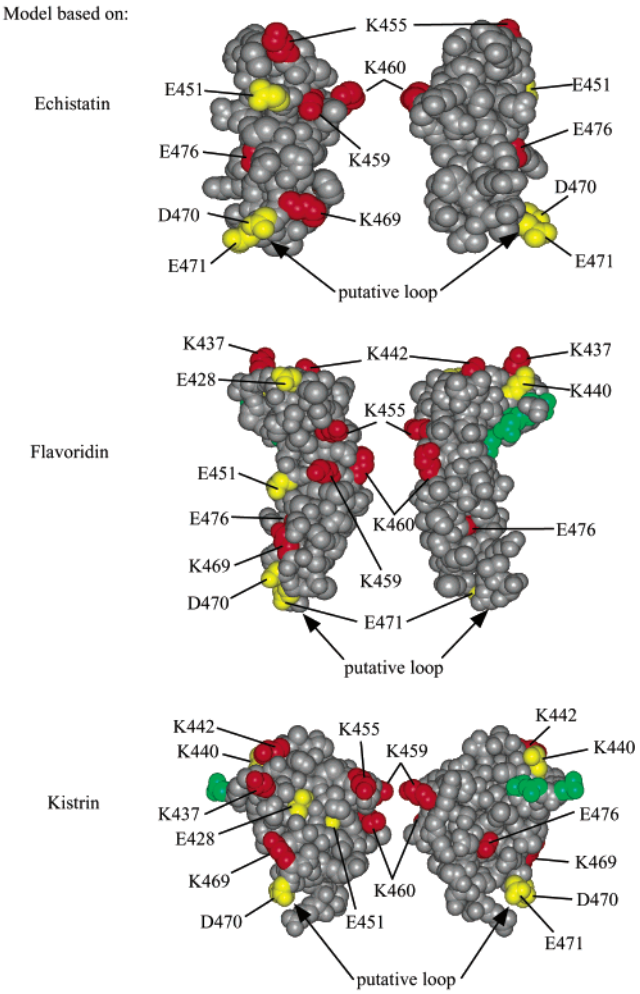

FIGURE 6: Structural models of the ADAM 28 disintegrin domain. Space-filled representations of models generated for the ADAM 28 disintegrin domain based upon three separate snake venom disintegrin NMR structures. Models were generated using software described in Experimental Procedures. The models are shown at 180° rotations. Residues colored red required more than 5-fold more protein when changed to alanine to achieve the same adhesion as the native sequence (Table 1). Residues shown in yellow required 2−5-fold more protein when changed to alanine to achieve the same adhesion as the native sequence. Residues shown to participate in mAb Dis 1-1 binding are shown in green on the flavoridin and kistrin models. The position of the putative disintegrin loop is indicated.

other disintegrin adhesion events (*28*). No effect on the ability of the rDis−Fc fusion protein to support Jurkat adhesion was observed when either of the acidic residues contained within the putative disintegrin loop was replaced with alanine when integrin activation was achieved by including 1 mM $MnCl_2$ in the adhesion buffer. Here we specifically activated only the β1 integrins by using mAb QE2E5, an anti-β1 activating monoclonal antibody (*41*). No differences were evident in the results obtained for the Asp^{473} mutant. However, altering the mechanism of activation affected whether alanine substitution at Glu^{471} elicited an effect. The various activation states of integrins that can be induced by exogenous activators and the effects of these multiple states on ligand recognition are well-documented for integrin $\alpha4\beta1$ (*48*, *49*). The extent of $\alpha4\beta1$-dependent cell adhesion to VCAM-1 or CS-1 can be altered depending upon whether the integrin is activated via an activating mAb, TS2/16, or through the presence of the divalent cation Mn^{2+} (*50*). Using two distinct exogenous activators for affinity modulation of the receptor could result in divergent activation states and account for the different results that are evident with alanine replacement of Glu^{471}. The extent of activation could directly influence the relative number of contacts or relative size of the receptor−ligand interface. Such sensitivity of the receptor−ligand complex to slight conformational changes of the receptor would give a more intricate and subtle way of regulating crucial adhesive events.

We have established that ADAM 28 interacts with the lymphocyte integrin $\alpha4\beta1$ via multiple charged residues dispersed through the linear sequence of the disintegrin domain. Molecular modeling studies suggest that the $\alpha4\beta1$ recognition site within ADAM 28 presents itself as an extended face of the disintegrin domain that may make multiple contacts with both integrin subunits. Evidence that the adhesive properties of a mammalian ADAM disintegrin domain are not restricted to residues contained within the putative disintegrin loop provides insight into the way this family of proteins that exhibit both adhesive and proteolytic functions act as integrin ligands.

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