ADAM disintegrin-like domain recognition by the lymphocyte integrins $\alpha 4\beta 1$ and $\alpha 4\beta 7$

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The ADAM (a disintegrin and metalloprotease) family of proteins possess both proteolytic and adhesive domains. We have established previously that the disintegrin domain of ADAM28, an ADAM expressed by human lymphocytes, is recognized by the integrin $\alpha 4\beta 1$. The present study characterizes the integrin binding properties of the disintegrin-like domains of human ADAM7, ADAM28 and ADAM33 with the integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$. Cell-adhesion assays demonstrated that, similar to ADAM28, the ADAM7 disintegrin domain supported $\alpha 4\beta 1$ -dependent Jurkat cell adhesion, whereas the ADAM33 disintegrin domain did not. The lymphocyte integrin $\alpha 4\beta 7$ was also found to recognize both disintegrin domains of ADAM7 and ADAM28, but not of ADAM33. This is the first demonstration that mammalian disintegrins are capable of interacting with $\alpha 4\beta 7$. All three disintegrin domains supported $\alpha 9\beta 1$ -dependent cell adhesion. Re-

cognition by both $\alpha 4\beta 1$ and $\alpha 4\beta 7$ of ADAM7 and ADAM28 was activation-dependent, requiring either the presence of Mn^{2+} or an activating monoclonal antibody for cell attachment. Charge-to-alanine mutagenesis experiments revealed that the same residues within an individual ADAM disintegrin domain function in recognizing multiple integrins. However, the residues within a specific region of each ADAM disintegrin-like domain required for integrin binding were distinct. These results establish that ADAM7 and ADAM28 are recognized by the leucocyte integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$. ADAM33 exclusively supported only $\alpha 9\beta 1$ -dependent adhesion.

Key words: a disintegrin and metalloprotease (ADAM), cell adhesion, disintegrin, integrin, lymphocyte, mutagenesis.

INTRODUCTION

ADAM (a disintegrin and metalloprotease) proteins are members of the metzincins that also include the related matrix metalloproteases. The defining feature of the ADAMs is a conserved modular domain organization consisting of a prodomain, a metalloprotease domain, a disintegrin-like domain, a cysteine-rich domain, an EGF (epidermal growth factor)-like domain, a transmembrane domain and a cytoplasmic domain [1,2]. The ADAMs comprise nearly 40 proteins with members being identified in a variety of mammalian species as well as lower eukaryotes such as *Caenorhabditis elegans*, *Xenopus* and *Drosophila* [3].

Although the metalloprotease domain of several ADAMs functions as a cell-surface sheddase [4,5], the functional relevance of the other ADAM domains remains unclear. The disintegrin-like domain of the ADAMs exhibits extensive homology with the small non-enzymic peptide constituents of snake venom that are potent integrin antagonists [6,7]. Multiple studies have reported integrin recognition of ADAM disintegrin-like domains. The mechanism of integrin recognition of snake venom disintegrins has been attributed to tripeptide sequences positioned on a structurally defined loop [8–10]. A similar region within ADAM disintegrin-like domains also exists. Although some charged residues within the putative disintegrin loop of the ADAMs are critical for integrin recognition [11–14], many of the residues that comprise the integrin recognition site are located outside of the putative loop [15].

Integrins are a cell–cell and cell–matrix receptor superfamily composed of non-covalently linked heterodimers. Integrins have

broad and significant biological roles in processes such as embryonic development, haematopoiesis, wound repair and immune response [16]. Recognition of multiple ADAM disintegrins by the same integrin family member has been demonstrated for the recognition of ADAM2, ADAM3 and ADAM9 by the integrin $\alpha 6\beta 1$ [13,14,17,18] and the recognition of ADAM2, ADAM3 and ADAM28 by $\alpha 4\beta 1$ [19,20], whereas $\alpha 9\beta 1$ was shown to recognize ADAM2, ADAM3, ADAM12 and ADAM15 [19,21], leading to the hypothesis that $\alpha 9\beta 1$ interacts with all prototypical ADAMs [12]. Promiscuity is not restricted to the receptor, but is additionally a property of the ADAM ligands. ADAM9 is recognized by $\alpha 6\beta 1$ and $\alpha v\beta 5$ [18,22] and ADAM15 binds $\alpha 9\beta 1$, $\alpha v\beta 3$ or $\alpha 5\beta 1$ [21,23,24]. A recent study suggests that multiple integrins expressed on the same cell facilitate cell adhesion to a single ADAM disintegrin domain [19]. This complex pattern of integrin–ADAM recognition is reminiscent of the well-established integrin–extracellular matrix and integrin–cellular counter receptor interactions.

Members of the integrin family can be segregated into five groups based on the sequence homology of α subunits and the similarity of ligand specificities [25]. The smallest grouping is comprised of the $\alpha 4$ and $\alpha 9$ integrins: $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$. These two integrin subunits are unique among all integrin α subunits in that they lack inserted A-domains and are not post-translationally processed into heavy and light chains [26]. The integrins $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$ share similar ligand specificity and functionality. The integrin $\alpha 4\beta 1$ functions in lymphocyte infiltration of tissue through VCAM-1 (vascular cell adhesion molecule-1) [27]. The integrin $\alpha 4\beta 7$ interacts with MAdCAM-1

Abbreviations used: ADAM, a disintegrin and metalloprotease; CHO cells, Chinese-hamster ovary cells; Dis-Fc, recombinant disintegrin–Fc fusion protein; mAb, monoclonal antibody; MAdCAM, mucosal addressin adhesion molecule.

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(mucosal addressin adhesion molecule-1) for proper homing of lymphocytes to lymphoid structures such as Peyer's patches to establish and maintain gut-associated immunity [28–30]. The integrin $\alpha 9 \beta 1$ has been demonstrated to be expressed by neutrophils and to mediate adhesion events that facilitate transendothelial migration [31,32].

As the biological role of the ADAMs becomes more evident, distinct functional classes of these ADAMs are recognizable. Two dominant classes are proteins involved in fertilization, which include ADAM1, ADAM2 and ADAM3 [33,34], and ADAM10, ADAM17, ADAM19 (MADDAM), ADAM28 and ADAM33 that have been implicated in immune functions. ADAM10 processes the chemokine fractalkine, which promotes monocytic chemotaxis [35]; ADAM17 cleaves tumour necrosis factor- α and L-selectin [36–38]; MADDAM is crucial for dendritic cell differentiation [39]; ADAM28 is localized to mature B-cells (R. D. Bowditch, unpublished work), cleaves a CD23 peptide [40] and binds $\alpha 4 \beta 1$ [20]; and ADAM33 has been genetically linked to asthma [41].

In the present study, we explore the possibility that the integrins $\alpha 4 \beta 1$, $\alpha 4 \beta 7$ and $\alpha 9 \beta 1$ recognize multiple mammalian disintegrin domains. We assessed the adhesive properties of the newly identified ADAM7 due to its homology and chromosomal proximity to ADAM28. We also evaluated the potential integrin recognition of ADAM33. On the basis of the current knowledge of these ADAM disintegrin domains and their respective integrin ligand properties established in this study, we propose that these ADAMs are functional ligands for $\alpha 4$ and $\alpha 9$ subclasses of the integrin family.

EXPERIMENTAL

Materials

The Jurkat cell line was obtained from the A.T.C.C. (Manassas, VA, U.S.A.). The RPMI 8866 cell line was a gift from Dr J. C. Wilkins (University of Manitoba, Manitoba, Canada). Both lymphoma cell lines were maintained in RPMI 1640, containing 10 mM Hepes, 1 mM sodium pyruvate, 10% (v/v) fetal calf serum, 1% L-glutamine and 1% penicillin–streptomycin. The parental K562 cell line was purchased from the A.T.C.C., and the $\alpha 4$ -K562 cell line was a gift from Dr R. Isberg (Tufts University School of Medicine, Boston, MA, U.S.A.). The $\alpha 9$ -transfected CHO cells (Chinese-hamster ovary cells) were described previously [32]. The anti-integrin mAbs (monoclonal antibodies) PIH4 (anti- $\alpha 4$), 44H6 (anti- $\alpha 4$), 6S6 (anti- $\beta 1$), YFC118.3 (anti- $\beta 2$) and Y9A2 (anti- $\alpha 9 \beta 1$) were purchased from Chemicon International (Temecula, CA, U.S.A.). The anti- $\beta 7$ mAb FIB 27 was obtained from BD PharMingen (San Diego, CA, U.S.A.). The anti- $\beta 1$ mAb AIB2 was kindly provided by Dr C. Damsky (University of California, San Francisco). The anti- $\beta 1$ -activating mAb QE2E5 [42] was affinity-purified from supernatants harvested from a hybridoma cell line provided by Dr R. Faull (Royal Adelaide Hospital, Adelaide, South Australia, Australia). The anti- $\beta 7$ -activating mAb 2G3 [43] and recombinant soluble MAdCAM-Ig were generously supplied by Dr T. Yednock (Elan Pharmaceuticals, San Francisco, CA, U.S.A.). The CS-1 peptide (EILDVPST) was purchased from the American Peptide Company (Sunnyvale, CA, U.S.A.).

Recombinant disintegrin fusion proteins

Methods for the generation of the ADAM28 Dis-Fc (recombinant disintegrin-Fc fusion protein) construct were described previously [15,20]. DNA fragments of ADAM7 (1121–1784 bp) and

ADAM33 (1202–1615 bp) containing the full-length disintegrin domain coding regions were obtained by nested PCR of a human cDNA lymph node and liver library (ClonTech, Palo Alto, CA, U.S.A.) respectively. By performing PCR overlap extension reactions, the full-length sequence encoding the disintegrin domains of ADAM7 (residues Tyr³⁹⁷–Gly⁴⁹⁷) and ADAM33 (residues Ala⁴¹⁸–Gly⁵¹⁰) was annealed in frame with a 5'-GP67 secretion signal sequence and a 3' human IgG3 Fc-fusion tag. The human IgG3 Fc heavy chain lacked the hinge region to eliminate Fc-mediated dimerization of the recombinant proteins. PCR products were subcloned into the TOPO pIB/V5-His vector (Invitrogen, Carlsbad, CA, U.S.A.). ADAM12 Dis-Fc was generated as discussed for ADAM7 and ADAM33 by using a nested PCR approach on a human cDNA liver library. The recombinant protein contained the entire ADAM12 disintegrin domain spanning residues Asn⁴¹⁴–Gly⁵¹⁹. Charge-to-alanine substitution mutants were produced with the Quik Change site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.). Synthetic oligonucleotides were obtained from the Molecular Biology Resource Facility at The University of Oklahoma Health Science Center or Sigma-Genosys (Woodlands, TX, U.S.A.). After sequence verification, recombinant Dis-Fc proteins were produced by stable transfection of High 5 cells with CellFectin (Invitrogen). Conditioned media were harvested, poly(ethylene glycol)-concentrated and applied on to a Protein G–Sepharose column (Amersham Biosciences, Uppsala, Sweden). Recombinant protein was eluted by the addition of 100 mM citric acid (pH 3.0), and column fractions were immediately neutralized with 1 M Tris/HCl (pH 9.0). All recombinant disintegrin proteins used in the present study migrated as a single band when evaluated by SDS/PAGE under reduced and non-reduced conditions, suggesting that the purified recombinant disintegrin domains did not possess any gross structural perturbations resulting in aberrant disulphide bond formation.

Cell-adhesion assay

The assay utilized was modified from that previously described for demonstrating cell adhesion to immobilized fibronectin [44]. For Jurkat and RPMI 8866 cell-adhesion experiments, recombinant disintegrin domains were immobilized on Immulon-2 96-well plates (Dynatech Laboratories, Chantilly, VA, U.S.A.) by incubating desired concentrations of recombinant protein in 0.1 M NaHCO₃ (pH 8.4) overnight at 4°C in a total volume of 100 μ l. Wells were subsequently blocked with 2% (w/v) BSA in 0.1 M NaHCO₃ (pH 8.4) at room temperature (22°C) for 1 h. Cells were washed three times with Hepes-Tyrodé's buffer (5 mM Hepes, 150 mM NaCl, 12 mM NaHCO₃, 2.6 mM KCl, 5 mM D-glucose, 0.2 mg/ml BSA, 0.5 mM MgCl₂ and 1 mM CaCl₂). Where indicated, the buffer additionally contained 1 mM MnCl₂. After washes, cells were adjusted to 2×10^6 cells/ml in Hepes-Tyrodé's containing 10 μ g/ml of the appropriate activating mAb: QE2E5 (anti- $\beta 1$) or 2G3 (anti- $\beta 7$). CHO cell-adhesion assays were as described for Jurkat cells except that cells were resuspended to 1×10^6 cells/ml in Hepes-Tyrodé's buffer that lacked exogenous integrin activators. Aliquots of 100 μ l of the cell suspension were incubated with immobilized substrates for 1 h at 37°C in an atmosphere of 5% CO₂. For inhibition experiments, inhibitors were suspended in Hepes-Tyrodé's buffer at twice the final desired concentration (50 μ l/well) and subsequently diluted with cells (50 μ l/well) at double the final concentration. Non-adherent cells were displaced with three consecutive washes of Hepes-Tyrodé's buffer. Adherent cells were disclosed by colorimetric assessment of endogenous acid phosphatase activity by the addition of 100 μ l/well of 1% Triton X-100, 50 mM sodium

Figure 1 The disintegrin domain of ADAM7 facilitates integrin $\alpha 4 \beta 1$ -dependent cell adhesion

(a) Alignment of the primary amino acid sequences of the disintegrin domains of human ADAM7, ADAM28 and ADAM33. Identities of ADAM28 with ADAM7 and ADAM33 disintegrin domains are shaded, with the respective homology of ADAM7 and ADAM33 to ADAM28 displayed at the far left. The predicted integrin $\alpha 9 \beta 1$ recognition motif is indicated by thick underlining and contains the putative disintegrin loops. Arrows indicate charged residues selected for alanine substitution in the ADAM7 and ADAM28 disintegrin domains. (b) Microtitre wells were coated with the indicated concentrations of ADAM7 (■), ADAM28 (○) or ADAM33 Dis-Fc (▲) recombinant proteins. ADAM7 and ADAM33 Dis-Fc contained the complete disintegrin domains Tyr³⁹⁷-Gly⁴⁹⁷ and Ala⁴¹⁶-Gly⁵¹⁰ respectively and ADAM28 Dis-Fc as described previously [15,20]. The T-lymphoma cell line Jurkat (2×10^5 cells/well) was added to wells in HEPES/Tyrosine's buffer containing 1 mM CaCl₂, 0.5 mM MgCl₂ and 10 μ g/ml QE2E5 mAb for exogenous activation of the $\beta 1$ integrins. The number of adherent cells per well was quantified based on a standard curve generated by assessing the endogenous acid phosphatase activity as described in the Experimental section. Adherent cells/well = adherent cells_(recombinant protein) - adherent cells_(BSA). Results shown are the means $\pm$ S.D. for a representative experiment performed in triplicate. (c) Microtitre wells were coated with 10 μ g/ml of purified, recombinant ADAM28 (white bars) or ADAM7 Dis-Fc (black bars). Jurkat cells were added to wells as described previously in a buffer containing 1 mM MnCl₂. Cells were incubated with the recombinant ligands in the presence of 5 μ g/ml of various integrin subunit mAbs or with 100 μ g/ml of the $\alpha 4 \beta 1$ ligand mimetic CS-1 peptide. Results are expressed as the average percentage inhibition $\pm$ S.D. for a representative experiment performed in triplicate. Percentage inhibition = $\{1 - [\text{adherent cells}_{(\text{inhibitor})} - \text{adherent cells}_{(\text{BSA})}] / [\text{adherent cells}_{(\text{no inhibitor})} - \text{adherent cells}_{(\text{BSA})}]\} \times 100$. (d) $\alpha 4$ -K562 (●) or K562 (○) cells were added to microtitre wells (2×10^5 cells/well) coated with various concentrations of ADAM7 Dis-Fc in the presence of 10 μ g/ml QE2E5. Adherent cells/well = adherent cells_(recombinant protein) - adherent cells_(BSA). Results are expressed as the mean adhesion $\pm$ S.D. for a representative experiment performed in triplicate.

acetate (pH 5.0) and 6 mg/ml *p*-nitrophenyl phosphatase and incubating at 37°C for 2 h. After the addition of 50 μ l/well of 1 M NaOH, absorbance at 405 nm was determined. Calculation of the number of adherent cells/well was derived from a standard curve of absorbance values generated from a known number of cells.

Flow cytometry

RPMI 8866 cells were washed twice with PBS containing 0.1 % BSA. Cells (5×10^5) were incubated in a total volume of 200 μ l with the appropriate primary anti-integrin antibody (10 μ g/ml) for 30 min at 4°C. The cells were washed twice with PBS/BSA and incubated with either 10 μ g/ml goat anti-mouse IgG-specific FITC-conjugated antibody (Caltag Laboratories, Burlingame, CA, U.S.A.) or with goat anti-rat IgG-specific FITC-conjugated antibody (Chemicon International, Temecula, CA, U.S.A.). The cells were incubated with the secondary antibody for 30 min at 4°C. Cells were washed twice with PBS/BSA and immediately analysed on an FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA, U.S.A.).

RESULTS

The disintegrin domain of ADAM7 is an $\alpha 4 \beta 1$ ligand

We have established previously that the disintegrin domain of ADAM28, a disintegrin expressed by mature B-lymphocytes (R. D. Bowditch, unpublished work), is recognized by the integrin $\alpha 4 \beta 1$ [20]. In the present study, we examined the potential of the homologous ADAM7 and ADAM33 disintegrin domains to facilitate $\alpha 4 \beta 1$ -dependent cell adhesion (Figure 1a).

The recombinant disintegrin domains of ADAM7 (ADAM7 Dis-Fc) and ADAM33 (ADAM33 Dis-Fc) were initially tested for their ability to mediate Jurkat cell adhesion (Figure 1b). As reported previously, the disintegrin domain of ADAM28 facilitated Jurkat cell adhesion in a dose-dependent and saturable manner when isolated (ADAM28 Dis-Fc; Figure 1b) or in the context of other domains (Dis-Cys-EGF-Fc) [20]. A similar but more apparent trend was observed with the ADAM7 Dis-Fc (Figure 1b). Whereas wells coated with 5 μ g/ml of the ADAM28 disintegrin domain exhibited a maximal 47.5 ± 3.7 % adhesion of the total input cells, the same concentration of recombinant ADAM7 disintegrin resulted in 88.5 ± 3.0 % adhesion. Considerably lower

Figure 2 The disintegrin domains of ADAM28 and ADAM7 are recognized by the immunological integrin $\alpha 4\beta 7$

(a) Flow-cytometric analysis of integrin subunit expression on RPMI 8866 cells. RPMI 8866 cells were incubated with the primary mAbs 44H6 (anti- $\alpha 4$), Y9A2 (anti- $\alpha 9\beta 1$), 6S6 (anti- $\beta 1$), YFCII8.3 (anti- $\beta 2$) and FIB27 (anti- $\beta 7$). After washing, the bound primary antibody was disclosed with appropriate secondary FITC-conjugated antibody (unfilled curve) as described in the Experimental section. Experiments containing only secondary conjugated antibody served as negative controls (filled curve). (b) Adhesion assays were performed as described previously by adding RPMI 8866 cells (2×10^5 cells/well) to microtitre wells coated with various concentrations of purified, recombinant ADAM7 (■), ADAM28 (○) or ADAM33 Dis-Fc (▲). Cells were suspended in HEPES-Tyrod's buffer containing 1 mM CaCl_2 , 0.5 mM MgCl_2 and 10 $\mu\text{g/ml}$ of the anti- $\beta 7$ activating mAb 2G3. Adherent cells/well = adherent cells_(recombinant protein) – adherent cells_(BSA). Results shown are the average $\pm$ S.D. for a representative experiment performed in triplicate. (c) RPMI 8866 cells (2×10^5 cells/well) suspended in a buffer containing 1 mM MnCl_2 were added to microtitre wells coated with 10 $\mu\text{g/ml}$ of the indicated recombinant ADAM28 (white bars) or ADAM7 Dis-Fc (black bars) in the presence of 5 $\mu\text{g/ml}$ of either function-blocking or non-function-blocking mAbs that recognize distinct integrin subunits. Results are expressed as the average percentage inhibition $\pm$ S.D. for a representative experiment performed in triplicate. Percentage inhibition = $\{1 - [\text{adherent cells}_{(\text{inhibitor})} - \text{adherent cells}_{(\text{BSA})}] / [\text{adherent cells}_{(\text{no inhibitor})} - \text{adherent cells}_{(\text{BSA})}]\} \times 100$.

concentrations of the disintegrin domain of ADAM7 were required to generate measurable levels of Jurkat cell adhesion when compared with ADAM28. As shown in Figure 1(b), no detectable cell adhesion was attained with ADAM33 Dis-Fc at the highest concentration tested (5 $\mu\text{g/ml}$).

To establish that Jurkat cell adhesion to the ADAM7 Dis-Fc was $\alpha 4\beta 1$ -dependent, function-blocking anti-human mAbs to selected α and β integrin subunits were used as inhibitors in the adhesion assay. Similar to the recombinant ADAM28 disintegrin domain, Jurkat cell adhesion to the ADAM7 Dis-Fc was inhibited by the anti- $\alpha 4$ and anti- $\beta 1$ function-blocking mAbs, indicating that the observed adhesion required $\alpha 4\beta 1$ (Figure 1c). The non-inhibitory anti- $\alpha 4$ (mAb), 44H6, did not substantially block Jurkat cell adhesion to ADAM7 Dis-Fc. Integrin $\alpha 4\beta 1$ dependence was supported by the potent inhibition of Jurkat cell adhesion to ADAM7 Dis-Fc by the fibronectin CS-1-derived peptide (EILDVPST; Figure 1c). Stable transfection of the human $\alpha 4$ -subunit cDNA into the K562 erythroleukaemic cell line conferred the ability to adhere to ADAM7 Dis-Fc, establishing that adhesion was directly dependent on the presence of the $\alpha 4\beta 1$ integrin (Figure 1d). The $\alpha 4$ -K562 cells exhibited similar adhesion trends

as the Jurkat cell line when the adhesion levels of ADAM28 and ADAM7 Dis-Fc were compared (results not shown).

The disintegrin domains of ADAM7 and ADAM28 support $\alpha 4\beta 7$ -dependent cell adhesion

Since the integrins $\alpha 4\beta 7$ and $\alpha 4\beta 1$ exhibit similar ligand recognition specificities and share a common α -subunit, recognition of the ADAM28, ADAM7 and ADAM33 disintegrin domains by $\alpha 4\beta 7$ was investigated. For these experiments, the B-cell-derived RPMI 8866 cell line was utilized. This cell line is classified as an $\alpha 4$ high, $\beta 7$ high, $\beta 1$ low expressing cell line, which was verified by flow cytometry (Figure 2a).

The RPMI 8866 cells attached in a dose-dependent and saturable manner to both the ADAM7 and ADAM28 Dis-Fc, whereas no significant adhesion was observed with ADAM33 Dis-Fc (Figure 2b). Integrin specificity was demonstrated by using function-blocking mAbs to the integrin subunits that would potentially be involved in this recognition (Figure 2c). Only function-blocking mAbs to the $\alpha 4$ and $\beta 7$ integrin subunits significantly decreased RPMI 8866 cell adhesion to ADAM28 and ADAM7

Figure 3 Recognition of the disintegrin domains of ADAM7 and ADAM28 by $\alpha 4\beta 1$ and $\alpha 4\beta 7$ integrins is activation-dependent

(a) Jurkat cells (2×10^5 /well) suspended in HEPES-Tyrode's buffer containing either 1 mM MnCl₂, 10 μ g/ml of the anti- $\beta 1$ -activating antibody QE2E5 or only 1 mM CaCl₂ and 0.5 mM MgCl₂ were added to wells coated with 10 μ g/ml each of ADAM7 (black bars) or ADAM28 Dis-Fc (white bars). (b) RPMI 8866 cell adhesion to 10 μ g/ml each of ADAM7 (black bars) or ADAM28 Dis-Fc (white bars) or 2.5 μ g/ml MAdCAM-Ig (hatched bars) was investigated in HEPES-Tyrode's buffer containing 1 mM MnCl₂, 10 μ g/ml of the anti- $\beta 7$ -activating antibody 2G3 or only 1 mM CaCl₂ and 0.5 mM MgCl₂. $\alpha 4\beta 7$ specificity was demonstrated for ADAM7 and ADAM28 by including 10 μ g/ml MAdCAM-Ig in solution with the 2G3-activating antibody. Results shown in (a, b) are the average $\pm$ S.D. for a representative experiment conducted in triplicate. Adherent cells/well = adherent cells(recombinant protein) – adherent cells(BSA).

Dis-Fc. Function-blocking mAbs to the $\beta 1$ and $\beta 2$ integrin subunits and the non-function-blocking anti- $\alpha 4$ mAb 44H6 had no significant inhibitory effects. Furthermore, the $\alpha 4\beta 7$ recombinant ligand MAdCAM-Ig inhibited the adhesion of RPMI 8866 cells to both ADAM28 and ADAM7 Dis-Fc (Figure 3b). In contrast with the results obtained with Jurkat cells, ADAM28 Dis-Fc showed significantly higher levels of adhesion with RPMI 8866 cells when compared with the ADAM7 Dis-Fc, suggesting that the ADAM28 disintegrin domain has a higher avidity for $\alpha 4\beta 7$ than the ADAM7 disintegrin domain.

$\alpha 4\beta 1$ and $\alpha 4\beta 7$ -dependent adhesion to ADAM7 and ADAM28 disintegrin domains requires integrin activation

In adhesion experiments with the two non-adherent cell lines described above, the activating mAbs QE2E5 (anti- $\beta 1$) or 2G3 (anti- $\beta 7$) were used. We have established previously that $\alpha 4\beta 1$ recognition of the disintegrin domain of ADAM28 depends on integrin activation. Similarly, Jurkat cell adhesion to the disintegrin

domain of ADAM7 required either the presence of the mAb QE2E5 or 1 mM MnCl₂ (Figure 3a). It was also demonstrated that $\alpha 4\beta 7$ -mediated RPMI 8866 cell adhesion to the disintegrin domains of ADAM7 or ADAM28 required exogenous integrin activation (Figure 3b). This was distinct from the results obtained with immobilized MAdCAM-Ig.

Homologous disintegrin domains utilize distinct but overlapping residues for integrin recognition

Six different charge-to-alanine disintegrin-loop mutants of ADAM7 and ADAM28 Dis-Fc were generated for addressing integrin recognition mechanisms utilized by homologous mammalian disintegrin domains (Figure 1a). In the present study, the mutagenesis was focused within the putative disintegrin loop, a region previously implicated in adhesion to several mammalian disintegrins, which also encompasses the predicted $\alpha 9\beta 1$ integrin recognition motif R(X6)DLPEF [12].

Six disintegrin-loop mutants of ADAM7 Dis-Fc were evaluated for their ability to support $\alpha 4\beta 1$ -dependent cell adhesion when compared with the native domain (Figure 4a). Only the conserved Arg⁴⁶¹/Arg⁴⁶⁶ and Lys⁴⁶⁴/Lys⁴⁶⁹ residues of ADAM7 and ADAM28 respectively exhibited similar effects when alanine substitution mutants were compared. Even though Asp⁴⁶⁵, Glu⁴⁶⁶ and Glu⁴⁷¹ of ADAM7 Dis-Fc were not critical for Jurkat cell adhesion, the homologous residues were required for maintaining the $\alpha 4\beta 1$ ligand properties of the ADAM28 disintegrin domain [15]. As with many other mammalian disintegrins, the Asp⁴⁶⁸ of ADAM7 was crucial in facilitating integrin-dependent cell adhesion [12,13].

A similar trend was evident when the ADAM7 and ADAM28 Dis-Fc charge-to-alanine mutants were tested for their relative ability to mediate $\alpha 4\beta 7$ -dependent cell adhesion (Figure 4b). Similar to the $\alpha 4\beta 1$ recognition of ADAM28 [15], alanine substitution of residues Lys⁴⁶⁹, Asp⁴⁷⁰, Glu⁴⁷¹ and Glu⁴⁷⁶ significantly decreased $\alpha 4\beta 7$ -dependent cell adhesion to ADAM28 Dis-Fc. However, alanine substitution of the homologous residues in ADAM7 did not affect $\alpha 4\beta 7$ -dependent cell adhesion. As observed for $\alpha 4\beta 1$ -dependent adhesion, the Asp⁴⁶⁸ in ADAM7 Dis-Fc negatively affected $\alpha 4\beta 7$ -dependent adhesion (Figure 4a). Interestingly, the homologous Asp⁴⁷³–Ala substitution within ADAM28 Dis-Fc had no effect on either $\alpha 4\beta 1$ or $\alpha 4\beta 7$ interaction.

The disintegrin domains of ADAM7, ADAM28 and ADAM33 are recognized by the integrin $\alpha 9\beta 1$

It has been hypothesized that $\alpha 9\beta 1$ integrin is a receptor for prototypical ADAMs [12]. Transfected CHO cells expressing human $\alpha 9$ subunit adhered to recombinant ADAM28, ADAM7 and ADAM33 Dis-Fc proteins, whereas mock-transfected CHO cells did not (Figure 5). Adhesion of CHO cells to $\alpha 9$ was also inhibited with Y9A2, a function-blocking anti- $\alpha 9\beta 1$ mAb (Figure 5). Results resembled levels attained with ADAM12 Dis-Fc, a disintegrin that has been previously identified as an $\alpha 9\beta 1$ ligand [21].

To define further the molecular mechanism involved in integrin recognition of mammalian disintegrins, we tested our ADAM7 and ADAM28 alanine substitution mutants for the ability to support $\alpha 9\beta 1$ -mediated cell adhesion (Figure 6). As with $\alpha 4\beta 1$ and $\alpha 4\beta 7$ recognition of ADAM28, substitution of Lys⁴⁶⁹, Asp⁴⁷⁰, Glu⁴⁷¹ and Glu⁴⁷⁶ with alanine resulted in decreased cell adhesion when compared with the native sequence. Only replacement of Asp⁴⁶⁸ with alanine within ADAM7 Dis-Fc elicited an adhesive defect.

Figure 4 Residues required for integrin $\alpha 4 \beta 1$ - and $\alpha 4 \beta 7$ -mediated cell adhesion to two homologous disintegrin domains

(a) Microtitre wells were coated with 5 μ g/ml of either native (white bar) or the indicated charge-to-alanine mutant of (black bars) ADAM7 or ADAM28 Dis-Fc. Jurkat cells were added as described previously in the presence of 10 μ g/ml QE2E5. Adhesion assays were performed as described in the Experimental section, and results shown are the average $\pm$ S.D. for three independent experiments each performed in triplicate. Percentage adhesion = [adherent cells_(native or mutant rDis-Fc) - adherent cells_(BSA)] / [adherent cells_(native rDis-Fc) - adherent cells_(BSA)] $\times$ 100. (b) RPMI 8866 cell adhesion to 5 μ g/ml of native ADAM7 or ADAM28 Dis-Fc (white bar) or charge-to-alanine mutants of ADAM7 or ADAM28 Dis-Fc (black bars) was evaluated in the presence of 10 μ g/ml of 2G3. Results shown are the average $\pm$ S.D. for three independent experiments each performed in triplicate. Percentage adhesion = [adherent cells_(native or mutant rDis-Fc) - adherent cells_(BSA)] / [adherent cells_(native rDis-Fc) - adherent cells_(BSA)] $\times$ 100.

DISCUSSION

Results of the present study established that the homologous disintegrin-like domains of human ADAM28 and ADAM7 are recognized by the leucocyte integrins $\alpha 4 \beta 1$, $\alpha 4 \beta 7$ and $\alpha 9 \beta 1$. As a model system for addressing the integrin ligand properties of ADAMs, disintegrin domains were recombinantly produced as Fc-fusion (Dis-Fc) proteins. This approach was based on previous studies demonstrating that the adhesive properties of isolated ADAM disintegrin domains are the same as domains expressed in the context of additional ecto-domains [18,20,21,23,24,45]. Cell adhesion to ADAM28 or ADAM7 Dis-Fc required exogenous activation of the receptor by inclusion of $MnCl_2$ or an activating mAb. Charge-to-alanine mutagenesis studies demonstrated that the same residues within specific ADAM disintegrin domains are used for the recognition of multiple integrins. The residues required for integrin binding, at least within the putative disintegrin loop, of each ADAM disintegrin domain differ. The disintegrin domain of human ADAM33 differs from ADAM7 and ADAM28 in that it is recognized by $\alpha 9 \beta 1$, but not by $\alpha 4 \beta 1$ or $\alpha 4 \beta 7$.

It is apparent that particular ADAMs may be recognized by multiple integrins. Multi-receptor binding is common among

Figure 5 The integrin $\alpha 9 \beta 1$ functions as a receptor for multiple mammalian disintegrin domains

Adhesion of CHO cells (1×10^5 cells/well) expressing the human $\alpha 9$ integrin subunit (■) or mock-transfected cells (○) to various recombinant mammalian disintegrin domains was examined in a concentration-dependent manner. The $\alpha 9 \beta 1$ function-blocking mAb Y9A2 was included at 5 μ g/ml at the highest ligand concentration (▲). Adhesion assays were performed as described previously in Hepes-Tyrosine's buffer containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$ with no exogenous integrin activators. ADAM12 Dis-Fc possessed the entire ADAM12 disintegrin domain spanning residues Asn⁴¹⁴-Gly⁵¹⁹. Results shown are the average $\pm$ S.D. for a representative experiment performed in triplicate.

Figure 6 Mutagenesis analysis of ADAM7 and ADAM28 disintegrin domains as ligands for the integrin $\alpha 9 \beta 1$

Microtitre wells were coated with 10 μ g/ml of native ADAM7 or ADAM28 Dis-Fc (white bars) or an appropriate charge-to-alanine mutant (black bars). $\alpha 9$ -CHO cells were added (1×10^5 cells/well) to wells and the extent of adhesion was determined as previously mentioned. Results shown are the average $\pm$ S.D. for three independent experiments each performed in triplicate. Percentage adhesion = [adherent cells_(native or mutant rDis-Fc) - adherent cells_(BSA)] / [adherent cells_(native rDis-Fc) - adherent cells_(BSA)] $\times$ 100.

integrin ligands [46]. Here, we have demonstrated that both the disintegrin domains of ADAM28 and ADAM7 are recognized by the lymphocyte integrins $\alpha 4 \beta 1$ and $\alpha 4 \beta 7$. In contrast with $\alpha 4 \beta 1$, the ADAM28 disintegrin domain consistently generated a higher level of $\alpha 4 \beta 7$ -dependent cell adhesion when compared with the ADAM7 disintegrin domain, suggesting an integrin preference for these ADAMs. This is the first demonstration that

mammalian disintegrins are capable of interacting with $\alpha 4\beta 7$. Only two other non-RGD-containing ADAMs, ADAM9 and ADAM23, have been reported to interact with non-very-late-antigen integrin family members [22,45]. Taken together, these findings suggest a broader and more diverse involvement of the integrin superfamily in ADAM's function. EC3, a heterodimeric disintegrin derived from the venom of *Echis carinatus*, is the only snake venom disintegrin shown to be a potent inhibitor of $\alpha 4\beta 7$ function [47]. However, there is no significant homology between EC3 and the disintegrin-like domains of ADAM28 or ADAM7 that would indicate a conserved mechanism for generating $\alpha 4\beta 7$ recognition. Interestingly, the disintegrin-like domain of human ADAM33 contains an MGD tripeptide sequence that is reminiscent of the MLD motif found in the snake venom disintegrins EC3, VLO5 and EO5 which is required for the recognition of integrins $\alpha 4\beta 1$ and $\alpha 9\beta 1$ [48]. However, ADAM33 was found to be not recognized by the integrin $\alpha 4\beta 1$. It should also be noted that the MGD sequence in human ADAM33 is not conserved in the mouse homologue of ADAM33.

It has been postulated recently that the integrin $\alpha 9\beta 1$ interacts with most prototypical ADAMs, a characteristic attributed to a highly conserved disintegrin loop motif [12]. This prediction is supported by current evidence that the disintegrin domains of ADAM2 and ADAM3 interact with $\alpha 9\beta 1$ [19]. We have expanded these results by demonstrating that ADAM7, ADAM28 and ADAM33 Dis-Fc are also recognized by $\alpha 9\beta 1$. Even though the putative $\alpha 9\beta 1$ recognition motif is common to all ADAMs recognized by $\alpha 9\beta 1$, our evidence suggests that each ADAM exhibits distinct integrin ligand properties. As demonstrated in the present study, some ADAMs exhibit both $\alpha 4\beta 1$ and $\alpha 9\beta 1$ ligand properties, and others such as ADAM33 are recognized exclusively by $\alpha 9\beta 1$.

The integrin binding surfaces of the homologous disintegrins of ADAM7 and ADAM28 are overlapping but distinct. Although similar regions of the domain interact with the integrin, the residues responsible within this region were unique for each ADAM. Even though the level of homology is not as extensive as with ADAM7 and ADAM28, similar results have been obtained with mutagenesis studies of the functionally linked ADAM2 and ADAM3 disintegrin domains [13,14,49]. When evaluating the binding properties of a particular ADAM, the same residues comprised the molecular contacts for multiple integrins, suggesting a highly conserved binding interface specific for each ADAM. Even though both $\alpha 4$ ligands require a conserved aspartic residue positioned in an extended CD loop for integrin binding, slight structural differences between VCAM and MAdCAM account for the unique binding activities of each ligand [50,51]. Perhaps, similar differences exist in the architecture of ADAM7 and ADAM28, accounting for the distinct avidities for $\alpha 4\beta 1$ versus $\alpha 4\beta 7$ and differences in residue requirement for integrin interaction.

A common theme evident from the present study is that all the integrins involved have proven roles in the migration of leukocytes from the vasculature to sites in the periphery. In the light of our current model that proposes that integrins may regulate ectodomain shedding by binding ADAMs [2], it is interesting to speculate about the functional consequences of the interaction of this group of ADAMs with the $\alpha 4/\alpha 9$ subset of the integrin family. The mature B-cell expression pattern of ADAM28 and the expression of $\alpha 4\beta 7$ on a defined population of circulating memory B-cells [52] suggests that, in certain physiological settings, ADAM28 and $\alpha 4\beta 7$ would be expressed on the same subset of lymphocytes. Association of ADAM28 through the disintegrin domain with $\alpha 4\beta 7$ may target or sequester the sheddase, thus altering the repertoire of lymphocyte surface adhesion molecules or cytokines affecting lymphocyte activity.

The present study establishes that the disintegrin domains of ADAM28 and ADAM7 are ligands for a functionally related subset of integrins composed of $\alpha 4\beta 1$, $\alpha 4\beta 7$ and $\alpha 9\beta 1$. By analysing six different charge-to-alanine disintegrin-loop mutants of these newly identified integrin ligands, we present evidence that suggests that the recognition of the same mammalian disintegrin by multiple integrins utilizes a highly conserved integrin recognition interface. Even though the residues that constitute the molecular contacts of various ADAMs are distinct, it appears that the integrin interface on each individual domain is spatially overlapping. Additionally, we have demonstrated that the disintegrin domain of ADAM33 is capable of mediating $\alpha 9\beta 1$ -dependent cell adhesion.

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