Integrin $\alpha_5\beta_1$ and ADAM-17Interact inVitro andCo-localize in Migrating HeLa Cells*

Received for publication, January 8, 2004, and in revised form, February 11, 2004
Published, JBC Papers in Press, February 16, 2004, DOI 10.1074/jbc.M400180200

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Tumor necrosis factor (TNF)-$\alpha$-converting enzyme (TACE/ADAM-17) has diverse roles in the proteolytic processing of cell surface molecules and, due to its ability to process TNF$\alpha$, is a validated therapeutic target for anti-inflammatory therapies. Unlike a number of other ADAM proteins, which interact with integrin receptors via their disintegrin domains, there is currently no evidence for an ADAM-17-integrin association. By analyzing the adhesion of a series of cell lines with recombining fragments of the extracellular domain of ADAM-17, we now demonstrate a functional interaction between ADAM-17 and $\alpha_5\beta_1$ integrin in a trans orientation. Because ADAM-17-mediated adhesion was sensitive to RGD peptides and EDTA, and the integrin-binding site within ADAM-17 was narrowed down to the disintegrin/cysteine-rich region, the two molecules appear to have a ligand binding pocket. Intriguingly, ADAM-17 and $\alpha_5\beta_1$ were found to co-localize in both membrane ruffles and focal adhesions in HeLa cells. When confluent HeLa cell monolayers were wounded, ADAM-17 and $\alpha_5\beta_1$ were redistributed to the leading edge and co-localized, which is suggestive of a cis orientation. We postulate that the interaction of ADAM-17 with $\alpha_5\beta_1$ may target or modulate its metalloproteolytic activity.

The ADAMs (a disintegrin and metalloproteinase) are a large family of type I transmembrane glycoproteins, many of which possess both protein processing and cell adhesive activities (for reviews, see Refs. 1–4). Thirty-four ADAMs have been identified in the human genome, at least twelve of which are predicted to have metalloprotease activity. In addition, there are 19 ADAMs, called ADAMTS, that are not anchored to the membrane and have an extra thrombospondin domain. Although the proteolytic processing activity of ADAMs has been implicated in growth factor/cytokine/receptor release from cell surfaces, cell migration, and cell fate determination, the presence of non-functional metalloproteinase domains in a number of family members is suggestive of wider functionality. All ADAM proteins have a common multidomain structure, comprising an N-terminal pro-domain, and metalloproteinase, disintegrin, cysteine-rich, and epidermal growth factor domains. Many members of the family possess cytoplasmic domains with potential sites for binding signaling proteins.

Perhaps the best-characterized ADAM proteinase is ADAM-17 (also known as tumor necrosis factor-alpha (TNF-$\alpha$)-converting enzyme (TACE)), which was first identified through its ability to cleave the pro-inflammatory cytokine TNF-$\alpha$ from its membrane-bound precursor (for a review, see Ref. 5). Subsequently, the availability of mutant mice, recombinant protein, and cell-based assays for protein processing have shown ADAM-17 to be involved in the cleavage of a wide variety of ligands and receptors, including $\beta$-amyloid precursor protein, pro-transforming growth factor-$\alpha$, CX3CL1, L-selectin, vascular cell adhesion molecule-1, Notch, TrkA nerve growth factor receptor, interleukin-6 receptor, and erbB4 epidermal growth factor receptor (6–16). Many of these molecules have well-established roles in inflammation, and because ADAM-17 is up-regulated in vivo during arthritis and experimental colitis (17–18), it has emerged as an important therapeutic target for inflammatory diseases (19). Mice bearing a proteolytically defective ADAM-17 gene display a similar phenotype to transforming growth factor-$\alpha$-null mice (precocious eye opening, stunted whiskers, and various epithelial defects (20–22)), suggesting that ADAM-17 also has an important role in embryonic development. Recently, mice bearing the ADAM-17 catalytic domain deletion were shown to prevent osteoclast recruitment and the formation of the narrow cavity in developing long bones (23).

All ADAMs contain a disintegrin domain that is related in sequence (and presumably structure) to snake venom disintegrins, and they are therefore potential integrin ligands. All snake venom disintegrins contain an RGD integrin-binding motif within a large, flexible loop, and use this to induce hemorraghe by antagonizing the pro-thrombotic platelet integrin $\alpha_{IIb}\beta_3$. Although only one ADAM (ADAM-15) contains an RGD sequence in an analogous position to the snake venom disintegrin motif, many other family members contain acidic motifs in its place that might mediate integrin binding. Through the use of protein-protein binding assays, synthetic peptide competition, and site-directed mutagenesis, a number of examples of
ADAM-integrin interactions have now been reported: ADAM-2 with αβ2 and αβ3, ADAM-3 with αβ3, ADAM-9 with αβ1 and αβ3, ADAM-15 with αβ1, and αβ3, ADAM-23 with αβ3, and ADAM-28 with αβ1, and a large number of ADAMs (1, 2, 3, 9, 12, and 15) with αβ3 (24–29).

Despite the biomedical importance of ADAM-17, there is currently no evidence that it interacts with an integrin. In this study, ADAM-17 was transiently introduced into human dermal fibroblasts for cell adhesion activity and found to support integrin αβ3-dependent attachment and spreading. ADAM-17 was also found to bind directly to purified αβ3. ADAM-17-integrin interactions were divalent cation-dependent, inhibited by synthetic RGD peptides, and mediated by the disintegrin/cysteine-rich domain region, suggestive of an interaction with the integrin ligand-binding pocket. Finally, ADAM-17 and αβ3 were found to co-localize in membrane protrusions during HeLa cell migration. Taken together, these data identify a novel integrin-ADAM interaction that has important implications for the known roles of ADAM-17 in inflammation.

EXPERIMENTAL PROCEDURES

Materials—The following monoclonal antibodies (mAbs) were either purchased or obtained as gifts: JAB18 (inhibitory mouse anti-human α2 integrin (30)); HP2/1 (inhibitory mouse anti-human α3 integrin; Francisco Sanchez-Madrid, Hospital de la Princesa, Universidad Autonoma de Madrid, Spain); 17E6 and 14D0FS (inhibitory mouse anti-human αα integrin; Simon Goodman, E. Merck, Darmstadt, Germany); P4C10 (inhibitory mouse anti-human β1 integrin; Invitrogen, Paisley, UK); 4B4 (inhibitory mouse anti-human β1 integrin; Coulter, Hialeah, FL); mAb13 (inhibitory rat anti-human β1 integrin), mAb16 (inhibitory rat anti-human αα integrin), and mAb11 (neutral rat anti-human αα integrin) (all from Ken Yamada, NIDCR, National Institutes of Health, Bethesda, MD); and JBS5 (inhibitory mouse anti-human α3 integrin; Serotec, Oxford, UK). A full-length cDNA clone of human α3 integrin was a gift from Ken Yamada and was subcloned into the pHRESneo2 vector (BD Biosciences Clontech, Cowley, UK).

Cell Culture—COS-1 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, UK). Human dermal fibroblasts and HeLa cells were provided by Karl Kaldar (University of Manchester, UK) and Philip Woodman (University of Manchester), respectively. COS-1 and HeLa cells were cultured in Dulbecco’s modified Eagle’s medium containing 0.11 g/liter sodium pyruvate, supplemented with 10% (v/v) fetal bovine serum, and 2 mM glutamine (Invitrogen). Cells were passaged every 3–4 days at a ratio of 1:10. Human dermal fibroblasts were cultured in minimal essential medium containing Earle’s salts, supplemented with 10% (v/v) fetal bovine serum, 2 mM glutamine, 1% (v/v) non-essential amino acids, 1 mM sodium pyruvate, and 1% (v/v) minimal essential medium vitamins (Invitrogen). Cells were passaged every 3–4 days at a ratio of 1:3.

Transfection of HeLa Cells—HeLa cells were transfected using 200 μg/ml EDTA, 500 μg/ml trypsin (BioWhittaker/Cambrex, Wokingham, UK) and plated overnight into 6-well culture plates (Costar, High Wycombe, UK) in complete medium. Cells were transfected with either 2 μg of α2-pIRES or pIRES vector alone or using LipofectAMINE Plus reagent (Invitrogen, Paisley, UK) according to the manufacturer’s instructions. 48 h post-transfection, cells were placed into 75-cm² flasks in growth medium supplemented with 1 mg/ml G418 (selection medium). 7–10 days after initial selection, G418-resistant colonies were harvested and expanded into routine culture. Cells expressing high levels of αβ3 integrin were selected by FACS.

Recombinant ADAM-17 Expression—cDNA fragments encoding full-length and truncated extracellular fragments of ADAM-17 (Fig. 1) were PCR-cloned from THP-1 mRNA into the pIRESneo2 vector (BD Biosciences Clontech) and expressed in COS-1 cells by electroporation (0.4-cm gap at 0.25 kV, 200 μF, 1.5 ms). Transfected cells were then cultured in medium cleared of immunoglobulins using protein G-Sepharose for 7 days. Recombinant protein was purified from the medium by protein A affinity chromatography, using 100 mM citric acid, pH 3.5, as eluant. All samples were neutralized with 1 M Tris-HCl, pH 8.5, and dialyzed against PBS. The enzymatic activity of recombinant ADAM-17 fragments was measured using a fluorescence resonance energy transfer-based assay. A peptide corresponding to the ADAM-17 cleavage sequence in pro-TNF-α (Ser-Pro-Leu-Ala-Gln-Ala-Val-Arg-Ser-Ser-Arg-Cys) containing an N-terminal fluorescent group (4,5-dimethyl-2-thiazolyl) and a C-terminal quenching group (4-(3-succinyl-1-yl)fluorescein-NH2) was incubated with ADAM-17 fragments diluted in cleavage buffer (50 mM Tris·HCl, pH 7.4, 2 mM CaCl₂, 0.1% (w/v) Triton X-100) for 16 h at 27 °C. Fluorescence was measured at 538 nm using an excitation wavelength of 485 nm. Any cleavage would be expected to separate the fluorescein and quenching groups and lead to increased fluorescence. The specificity of this reaction was tested using a peptidyl-hydroxamate inhibitor of TACE at 10 μM (compound 2α; prepared as described in Ref. 31).

Monoclonal Antibody Production—Anti-ADAM-17 mAbs were raised in BALB/c mice. Mice were immunized with 100 μg of ADAM-17-Fc and then boosted 1 month later with the same amount. One week before the boost, mice were given a final booster injection. Spleen cells were fused to 653 myeloma cells using 50% (w/v) polyethylene glycol. Hybridomas recognizing ADAM-17, and not the Fc tag, were identified by ELISA screening on ADAM-17-Fc and CD14-Fc, and dilution cloned. One clone, 34.D4, was grown in bulk, and purified by protein G affinity chromatography.

Cell Spreading and Attachment—For cell spreading assays, protein ligand was diluted into PBS containing divalent cations, and 100-μl aliquots were applied to the wells of flat-bottomed, 96-well tissue culture plates. After incubation at room temperature for 1 h, the wells were blocked for 1 h with 200 μl of 10 mg/ml heat-denatured (85 °C, 10 min) bovine serum albumin (BSA). Near-confluent 75-cm² flasks of human dermal fibroblasts were detached using trypsin/EDTA and quadruplicate with medium. Cells were resuspended in Eagle’s medium containing 25 mM HEPES and 2.5 mM glucose to a density 1 × 10⁶/ml, and 100-μl aliquots were added to each well. Cells were incubated at 37 °C for 1 h, then fixed with 10 μl of 50% (v/v) glutaraldehyde for 10 min. Percentage cell spreading was measured by phase contrast microscopy. For cell attachment assays, coating of wells and cell detachment were performed as described above, except that cells were resuspended to 2 × 10⁶/ml. After cell adhesion to ligand-coated wells, the plates were incubated at 37 °C for 20 min. Unattached cells were removed by aspiration, and the remaining cells were fixed with 100 μl of 5% (w/v) glutaraldehyde for 20 min. Wells were then washed three times with 300 μl of divalent cation-free PBS, and cells were extracted with 100 μl of 0.1% (w/v) crystal violet in 0.1% (w/v) HCl, 100 mM Na₂EDTA, and 0.1% (v/v) Triton X-100. The wells were washed extensively with water to remove excess crystal violet, and the stain was solubilized with 100 μl of 10% (v/v) acetic acid. The absorbance was measured at 570 nm using a 96-well plate reader.

Integrin-ligand Binding—The wells of a 96-well plate (Immulon-3, Dynatech, Chantilly, VA) were incubated overnight at room temperature with 100-μl aliquots of purified placental integrin αβ3 (32) diluted 1:300 in PBS containing divalent cations and were then blocked with 200 μl of 5% (w/v) BSA in TBS, pH 7.4, for 3 h at room temperature. After three washes with 300 μl of 1× McCl², 1 mg/ml BSA, TBS, pH 7.4 (wash buffer), 100-μl aliquots of ADAM-17-Fc in wash buffer were added to the plates at room temperature, and the wells were washed twice with 300 μl of wash buffer. Bound ADAM-17-Fc was detected by addition of 100-μl aliquots of 1:1000-diluted HRP-conjugated goat anti-human Ig secondary antibody (Sigma, Poole, UK) diluted in wash buffer. Following incubation at room temperature for 20 min, excess HRP conjugate was removed with four 300-μl washes of wash buffer, and 100-μl aliquots of 40 μl 2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) in 0.1 M sodium acetate, 0.05 M sodium phosphate, pH 5, 0.01% (v/v) H₂O₂ were added. The absorbance at 405 nm was measured using a plate reader.

Western Blotting—Samples were separated on 4–12% Bis/Tris SDS gels (Novex, Invitrogen) in MOPS buffer. Following transfer onto nitrocellulose, the filter was blocked in 5% (w/v) milk in PBS, 0.05% (v/v) Tween 20 overnight at 4 °C. Anti-ADAM-17 primary antibody was diluted to 10 μg/ml in 5% (w/v) milk in TBS, 0.05% (v/v) Tween 20 and incubated with the nitrocellulose for 1–2 h at room temperature with agitation. Following four 5-min washes in 20 ml of TBS, 0.05% (v/v) Tween 20, the nitrocellulose was washed with 10 ml of 0.1% (v/v) H₂O₂ solution, and the nitrocellulose was washed with 10 ml of TBS, 0.05% (v/v) Tween 20. HRP was then detected with 10 ml of chemiluminescence reagent for 2 min and exposed to Kodak Biomax photographic film in the dark.

Wounding and Immunofluorescence Microscopy—αβ3-transfected HeLa cells were plated onto glass coverslips at high density in selection medium and grown to confluence. Cell monolayers were wounded by a single scratch of a scalpel blade across the surface of the coverslips. After varying times, cells were fixed for 10 min in 4% (v/v)
paraformaldehyde in PBS. Fixed coverslips were blocked and permeabilized with 20% (v/v) normal donkey serum, 5% (v/v) heat-inactivated normal human serum, 0.25% (w/v) Triton X-100, 1 mg/ml BSA in PBS. Coverslips were then incubated for 1 h in 10 μg/ml 34.4D (anti-ADAM-17) and 10 μg/ml mAb11 (anti-5o) or 10 μg/ml mouse and rat IgG (Sigma), respectively. After five washes with PBS, coverslips were incubated with TRITC-conjugated donkey anti-rat and FITC-conjugated donkey anti-mouse secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:200 with PBS. Cells were analyzed using an Olympus IX70 microscope, with a 63 oil objective, and images were processed by constrained iterative deconvolution (DeltaVision, Applied Precision Instruments, Seattle, WA). 0.2-μm-thick z-sections are shown.

FACS—Confluent flasks of cells were detached using 5 mM EDTA in Hanks’ buffered saline solution at 37°C and resuspended in serum-containing medium to a density of 1 × 10^7/ml. 10 μg/ml primary antibody, diluted in 0.02% (v/v) sodium azide in divalent cation-free PBS, was added on ice for 45 min, and the cells washed three times with 300 μl of 1% (v/v) fetal bovine serum in divalent cation-free PBS. 1:200 FITC-conjugated anti-mouse secondary antibody in divalent cation-free PBS containing 10% (v/v) human serum was added on ice for 30 min, and then the cells were washed twice with 300 μl of divalent cation-free PBS containing 1% (v/v) FBS and once with 300 μl of divalent cation-free PBS. Cells were fixed with 100 μl of 2% (v/v) formaldehyde in divalent cation-free PBS. For each sample, 2 × 10^4 cells were counted using a FACScan flow cytometer (BD Biosciences, Cowley, Oxford, UK) at a flow rate of less than 200 events/s.

RESULTS

Expression of Recombinant ADAM-17-Fc—Although key functions have been established for the proteolytic activity of ADAM-17, its potential cell binding activity has not been investigated. Therefore, to determine whether ADAM-17 possesses adhesive activity, the full-length extracellular region of the molecule (amino acids 1–651; Fig. 1) was expressed as an Fc fusion protein and used as an adhesion substrate. The ADAM-17-Fc construct was expressed transiently in COS-1 cells and purified from culture medium using protein A affinity chromatography. Coomassie Blue staining following SDS-polyacrylamide gel electrophoresis under reducing conditions revealed a doublet of ~120 and 140 kDa indicative of the precursor and processed forms of ADAM-17 (Fig. 2A). Correct removal of the pro-domain at an intramolecular furin cleavage site situated between the pro- and metalloprotease domains was confirmed by N-terminal sequencing of the 120-kDa protein band. Under non-reducing conditions, a broad band of ~250 kDa was observed, which presumably corresponds to a mixture of disulfide-bonded Fc dimers of the 120- and 140-kDa proteins (Fig. 2A). Consistent with this conclusion, the 120-, 140-, and ~250-kDa bands all reacted with an

![Fig. 1. Domain organization of ADAM-17 and the recombinant ADAM-17-Fc, MP-Fc, and Dis-Fc constructs used in this study. From the N terminus, ADAM-17 contains pro- (Pro), metalloproteinase (MP), disintegrin (Dis), cysteine-rich (Cys), crambin, transmembrane, and cytoplasmic domains. The furin cleavage site is indicated by an arrow. The residues included in each recombinant construct are indicated in parentheses. All recombinant constructs contained a human IgG1 tag at the C terminus.](image)

![Fig. 2. A, SDS-PAGE analysis of recombinant ADAM-17-Fc. Purified ADAM-17-Fc was analyzed under non-reducing (lane 1) or reducing conditions (lane 2). B, Western blotting of ADAM-17-Fc (lanes 1 and 3) or CD14-Fc (lanes 2 and 4) with mouse anti-ADAM-17 mAb 34.4D under reducing (lanes 1 and 2) and non-reducing conditions (lanes 3 and 4). C, enzymatic activity of recombinant ADAM-17. The ability of ADAM-17-Fc (diamonds) and CD14-Fc (squares) to cleave a fluorescent Pro-TNF-α peptide was measured by fluorescence resonance energy transfer.](image)
anti-Fc antibody following Western blotting (data not shown) and with the anti-ADAM-17 mAb 34.4D (Fig. 2B).

As one approach to determine whether the ADAM-17-Fc protein was folded correctly, its metalloproteinase activity was measured. For this purpose, an assay based on cleavage of a fluorescently labeled 10-amino acid peptide corresponding to the ADAM-17 cleavage site in pro-TNF-α was employed. As shown in Fig. 2C, ADAM-17-Fc possessed dose-dependent proteolytic activity that was complete at only 0.5 μg/ml protein, consistent with an enzymatic reaction. A peptide-hydroxamate acid-competitive inhibitor of ADAM-17 (compound 2a) inhibited this cleavage completely at 10 μM, demonstrating the specificity of the ADAM-17 activity (data not shown). Recombinant CD14-Fc, employed as a negative control, gave no detectable cleavage of the pro-TNF-α substrate. Thus, recombinant ADAM-17-Fc was expressed and processed normally. The protein also possessed metalloproteinase activity, which is suggestive of correct folding.

ADAM-17 Supports Adhesion of Fibroblastic Cells—To examine whether ADAM-17 could support cell adhesion, both attachment and spreading assays were performed with human dermal fibroblasts. These cells were chosen because they express a wide range of extracellular matrix receptors. Efficient coating of the plastic substrate with ADAM-17-Fc was confirmed by anti-Fc ELISA assay (data not shown). ADAM-17-Fc supported fibroblast adhesion in a saturable, dose-dependent manner, whereas a CD14-Fc-negative control was unable to support adhesion above the BSA background (Fig. 3A). A maximal level of cell attachment of ~50% was observed at a coating concentration of 20 μg/ml ADAM-17-Fc, which was slightly lower than that of a 50-kDa fragment of fibronectin positive control (~70% attachment). Half-maximal attachment was observed at ~4 μg/ml ADAM-17-Fc and ~0.07 μg/ml 50-kDa fragment, which suggests that ADAM-17 interacts with the cell surface with lower affinity.

Similar results were obtained in cell spreading assays (Fig. 3B). Thus, ADAM-17-Fc supported dose-dependent, saturable spreading of dermal fibroblasts to a maximum of ~70%, compared with a level of ~95% obtained with the 50-kDa fragment of fibronectin. Half-maximal spreading was achieved at ~7 μg/ml (ADAM-17) and 0.11 μg/ml (50-kDa fibronectin). CD14-Fc and BSA controls were inactive. Taken together, these data indicate that ADAM-17 is able to promote both attachment of normal fibroblasts and that this adhesion is able to elicit a signaling response that results in cell spreading. In other studies, ADAM-17 was found to support the adhesion of a variety of other cell lines, including B16 melanoma, K562 erythroleukemia, and HCS-2/8 chondrosarcoma cells (data not shown).

Cell Adhesion to ADAM-17 Is Mediated by Integrin α5β1—Because ADAM-17 contains a disintegrin domain, integrins are candidate adhesion receptors that could be responsible for the observed cell adhesion and spreading onto ADAM-17-Fc. Integrin-ligand interactions are strongly divalent cation-dependent and EDTA-inhibitable, and, consistent with a role for integrins in ADAM-17 recognition, EDTA blocked dermal fibroblast adhesion to ADAM-17-Fc (Fig. 4A). Furthermore, when dermal fibroblasts were plated in cation-free PBS, cell attachment to ADAM-17-Fc was only observed when the buffer was supplemented with Mn2+ (70% attachment) or, to a lesser degree, with Mg2+ (25% attachment; Fig. 4A). Supplementation with Ca2+ did not support adhesion above BSA background levels. This pattern of cation dependence, in particular the potent stimulation by Mn2+, is consistent with integrin-dependent adhesion.

To identify integrin subunits potentially responsible for cell adhesion to ADAM-17-Fc, a series of inhibitory anti-integrin mAbs was screened for their ability to inhibit cell spreading onto 20 μg/ml ADAM-17-Fc (Fig. 4B). Antibodies directed against β1 (4B4, P4C10) or α5 (JBS5, mAb16) subunits reduced spreading by ~50 and ~70%, respectively. Inhibitory antibodies directed against α2 (JA218), α4 (HP2/1), and α7 (17E6, 14D9F8) had no effect. This pattern of inhibition was similar to that for cells spreading onto the 50-kDa fragment of fibronectin, although the degree of inhibition was lower (probably due to use of αc integrins in recognizing the 50-kDa fragment of fibronectin).

Because α5β1, α1β3, and all αi integrins recognize the RGD tripeptide motif in their ligands, short RGD-containing synthetic peptides can be used to inhibit ligand binding by competitively binding to the integrin ligand-binding pocket. To establish whether ADAM-17-Fc bound to α5β1 at the ligand binding pocket, 500 μM GRGDS peptide was included during fibroblast attachment onto 20 μg/ml ADAM-17-Fc (Fig. 4C). The non-functional control peptide GRDGS was included as a

![Fig. 3. Human dermal fibroblast attachment (A) and spreading (B) onto immobilized ADAM-17-Fc (diamonds), CD14-Fc (triangles), and the 50-kDa fragment of fibronectin (squares).](image-url)
negative control. Attachment to ADAM-17 Fc was inhibited by GRGDS by 85% and only slightly reduced by GRDGS. These results suggest that the binding of dermal fibroblasts to ADAM-17-Fc is RGD-dependent, and therefore that ADAM-17 binds at, or close to, the ligand-binding pocket of α2β1.

Finally, to determine whether ADAM-17 bound directly or indirectly to α2β1, the ability of ADAM-17-Fc to bind to purified placental α2β1 was tested in a solid phase assay (Fig. 5). ADAM-17-Fc bound to immobilized α2β1, but not BSA, in a dose-dependent manner, indicative of a direct interaction between the two proteins.

Localization of Adhesive Activity to the Disintegrin Domain of ADAM-17—To determine which domain of ADAM-17 was responsible for the ADAM-17-α2β1 interaction, two truncated ADAM-17 constructs were expressed (Fig. 1). Because it has been reported previously that the pro-domain is required for metalloproteinase domain folding (33), the first construct contained the signal peptide, pro-, and metalloproteinase domains...
These cells expressed high levels of ADAM-17 (as observed previously (34)). Although a large number of cell lines, HeLa cells were found to express using immunofluorescence microscopy. Following screening of cell lines, HeLa cells were stably transfected with full-length integrin α5β1/H9251 and integrin α5β1/H9252 cultures of HeLa cells, a striking co-localization of ADAM-17 with α5β1/H9251 and α5β1/H9252 on Dis-Fc was completely blocked by mAb13 (anti-β1) and α5 mAb (10A4). Thus, cell adhesion to ADAM-17 occurs via the disintegrin or Cys-rich domains in an integrin α5β1-dependent manner.

Co-localization of ADAM-17 and Integrin α5β1 in Migrating HeLa Cells—To test whether ADAM-17 and α5β1 associated in cells, either in cis or in trans, their distribution was examined using immunofluorescence microscopy. Following screening of a large number of cell lines, HeLa cells were found to express high levels of ADAM-17 (as observed previously (34)). Although these cells expressed α5 and β1, the levels of α5 were low for visualization by immunofluorescence, and therefore the cells were stably transfected with full-length α5 cDNA. In sparse cultures of HeLa cells, a striking co-localization of ADAM-17 and α5β1 was found in both ruffling membrane and in focal adhesions (Fig. 7). Co-localization was also observed in confluent cells, with both ADAM-17 and α5β1 exhibiting a peripheral distribution at cell-cell junctions (Fig. 8, A–C).

To examine the potential redistribution of ADAM-17 and α5β1 during migration, cell monolayers were wounded by scratching. ADAM-17-α5 co-localization was retained immediately after wounding, but after 2 h cells at the wound edge initiated membrane ruffling and elaborated protrusions into the wound space. ADAM-17 and α5 integrin co-localized specifically at the leading edge of these ruffles. Simultaneously, staining for both molecules in cell-cell junctions away from the wound edge was reduced (Fig. 8, D–F). By 4 h post-wounding, the cells at the edge of the wound had begun to migrate into the wound space. Migrating cells had less elaborate cell-junctions, and correspondingly more diffuse ADAM-17 and α5 integrin staining between cells proximal to the wound edge. Co-localization of ADAM-17 and α5 appeared strongest in membrane protrusions of cells at the leading edge (Fig. 8, G–I). After 6 h, the wound was nearly closed, but ADAM-17-α5 co-localization was retained at discrete points along the leading edge of cells at the wound edge (Fig. 8, J–L). At 8 h post-wounding, cell-cell contacts began to re-establish, and ADAM-17 and α5 integrin staining was co-localized in small foci between cells (Fig. 8, M–O). If the monolayer was left to fully heal, ADAM-17-α5 staining returned to its original cell-cell junction distribution (data not shown).

**DISCUSSION**

ADAM-17/TACE has a key role in membrane protein processing, especially pro-TNF-α, and thus is an important therapeutic target for various inflammatory conditions. In this study, we have utilized a recombinant soluble ADAM-17-Fc construct to explore its interactions with the integrin family of adhesion receptors. Our major findings are: (a) substrate-immobilized ADAM-17 is able to support the attachment and spreading of human dermal fibroblasts and a number of other cell types, (b) ADAM-17-dependent adhesion is mediated by the integrin α5β1, (c) integrin-binding activity resides in the disintegrin/cysteine-rich region of ADAM-17, (d) ADAM-17 binding is EDTA- and RGD peptide-inhibitable, and (e) ADAM-17 and α5β1 co-localize in cell-cell junctions and in membrane protrusions in migrating HeLa cells.

Previously, members of the ADAM family have been shown to bind to α5β1, α6β4, and αvβ3, and the RGD-containing ADAM-15 binds to α6β4 and αvβ3. In our studies, anti-αv and anti-β3 integrin antibodies inhibited cell spreading on ADAM-17-Fc to at least the same extent as on its ECM ligand fibronectin, and ADAM-17-Fc bound directly to purified α5β1. This suggests that ADAM-17 acts as a specific ligand for the integrin α5β1. By using fragments of ADAM-17, the interaction was shown to be mediated either by the ADAM-17 disintegrin domain or the cystein-rich domain, but we were unable to narrow the active site further, because the bacterially expressed disintegrin domain failed to fold (data not shown). If the disintegrin domain does contain the α5β1-binding site, as demonstrated for all previous ADAM-integrin interactions, then the identity of the residues contributing to the binding event is unclear. ADAM-17 lacks an RGD motif, indeed, it lacks any acidic residues in an analogous location to previously characterized integrin-binding sequences, and it is therefore conceivable that an alternative binding mode is employed.

A major issue for all ADAM-integrin interactions is whether they take place in cis (i.e. within the same cell) or in trans (i.e. between two cells). Although many interactions in trans orientation have been reported previously (e.g. 25, 28–29, 35–36), and its ability to promote cell adhesion suggests that this is also the case for ADAM-17, there is little evidence that a cis interaction could occur between an ADAM and an integrin. Although it is outside of the scope of this report, it will be important to follow-up our observation of co-localization of ADAM-17 and α5β1 at the leading edge of migrating cells to determine if they physically interact, and if so, how the...
binding is coordinated in relation to ligand occupancy and cell migration.

The interaction of ADAM-17 with the ligand-binding pocket of \( \alpha_\beta_1 \) has important implications for biological function, because it implies that a ternary integrin-ligand-ADAM complex cannot be formed. Based on the binding data in Fig. 5, the apparent affinity of ADAM-17 binding to \( \alpha_\beta_1 \) is \( \sim 200 \) nM, which is relatively weak compared with the \( \sim 5 \) nM affinity of fibronectin for \( \alpha_\beta_1 \) (37). Although it is conceivable that the comparatively low affinity of the ADAM-17-\( \alpha_\beta_1 \) interaction might enable a dynamic recruitment to, and release of, metalloproteinase activity at sites of cell-ECM adhesion, it is also possible that integrin-ADAM engagement modifies intracellular signaling through receptor cross-talk. Members of the tetr policeman family of cell surface proteins are well established as integrin-binding molecules and may have a role in integrin clustering and recycling. Recently, three members of this family have been reported to interact with ADAM-3 (38), raising the possibility that ADAMs may be co-clustered with integrins in an organized manner. The disintegrin-like and cysteine-rich

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**Fig. 6. Localization of the integrin-binding domain within ADAM-17.** A, human dermal fibroblast spreading on immobilized recombinant ADAM-17-Fc (squares), Dis-Fc (circles), and CD14-Fc (triangles). B, human dermal fibroblast spreading on recombinant ADAM-17-Fc (squares), MP-Fc (circles), and MAdCAM-1-Fc (triangles). C, anti-integrin mAb inhibition of human dermal fibroblast spreading on 20 \( \mu \)g/ml ADAM-17-Fc (closed bars) or 20 \( \mu \)g/ml Dis-Fc (open bars). mAbs 10A4 (neutral anti-\( \alpha_\beta_1 \)), mAb16 (inhibitory anti-\( \alpha_\beta_1 \)), and mAb13 (inhibitory anti-\( \beta_1 \)) were included during cell spreading at 20 \( \mu \)g/ml.
domains of recombinant jararhagin (a snake venom reproulysin metalloproteinase) have been shown to bind to the soluble ectodomain of αβ1 integrin in a divalent cation-independent manner in vitro (39). In cell-based assays, binding of the jararhagin disintegrin domain to fibroblasts expressing αβ1 caused these cells to up-regulate expression of active MMPs and αβ1 effects usually only mediated by the αβ1 ligand, type I collagen. The possibility that ADAM-17 interaction with αβ1 could generate signals that lead to up-regulation of pro-migratory gene expression is worth further attention.

The cytoplasmic tail of ADAM-17 has potential signaling motifs such as an SH3 and a PDZ3 domain that have been shown to recruit the protein-tyrosine phosphatase-H1 (PTPH1) (40) and the scaffolding protein synapse-associated protein 97 (SAP97) (41). The signaling potential of ADAMs is not well understood, however, ADAM-9 and ADAM-15 have been reported to interact with the SH3 domain-containing proteins, endophilin I and SH3PX1 (42), and ADAM-12 and ADAM-15 have been reported to bind to members of the Src tyrosine kinase family and the adaptor protein Grb2 (43, 44). Phosphorylation events associated with binding of SH3 domain-containing proteins to the cytoplasmic tails of ADAM proteins may provide a mechanism for selective regulation of ADAM signaling and function. Kang et al. (45) found that the p85 subunit of phosphatidylinositol 3-kinase bound to the cytoplasmic tail of ADAM-12, implicating ADAM-12 in the activation of phosphatidylinositol 3-kinase and the regulation of myoblast fusion. The role of ADAM-12 in myoblast fusion also provides additional clues as to the function of ADAM cytoplasmic tails. Two recent studies found that the C-terminal domain of ADAM-12 interacted with different α-actinin isoforms, and that this interaction was required for myoblast fusion (46, 47). Coupling of cell surface ADAMs to the cytoskeletal machinery via actin-binding proteins such as α-actinin may enable the cell to adapt to changes in tensile force generated by proteolytic cleavage of ADAM substrates and/or binding to cell adhesion partners. In addition, direct linkage of ADAM proteins to the cytoskeleton could allow co-ordination of signaling and morphological changes during cell fusion or migration. Because α-actinin is frequently observed in protruding membrane ruffles, similar to the structures observed here in HeLa cells, it is possible that ADAM-17 makes a contribution to the regulation of cytoskeletal architecture during migration. It will be of interest to investigate potential cytoplasmic interactions of the ADAM-17-αβ1 integrin complex.

If ADAM-17 acts as an integrin ligand in cis, indeed if any ADAM were to do this, then it is important to consider the
structural basis of the binding. The solution of the $\alpha_\beta$ integrin crystal structure raises the intriguing possibility that integrins are folded into a bent conformation (48), bringing the extracellular ligand-binding pocket proximal to the cell membrane. This would likely place the disintegrin and cysteine-rich domains of a cell-surface ADAM-17 molecule in a suitable position to bind a neighboring $\alpha_\beta$ integrin molecule. A potentially similar type of clustering between a membrane-anchored matrix metalloproteinase (MT1-MMP) and $\alpha_\beta$ integrin at the leading edge of migrating endothelial cells (49), and between MT1-MMP and $\beta$ integrins at the leading edge of fibrosarcoma cells migrating through a three-dimensional collagen matrix, has recently been reported (50). A further parallel is the cell surface association of tissue inhibitor of metalloproteinase-2 with MT1-MMP and the binding of tissue inhibitor of metalloproteinase-2 to ADAM-17 (51). As has been suggested for the MT1-MMP-integrin association, such cis interactions may serve a variety of functions: they may direct proteolytic activity to the leading edge of migratory cells, spatially restrict degradative activity, or facilitate the regulated turnover of ligands and/or receptors.

Alternatively, the proteolytic activity of ADAM-17 may be important during cell migration into a wound. A recent study showed that the disintegrin and cysteine-rich domains of ADAM 13 can bind the second heparin-binding domain of fibronectin and promote cell adhesion via activated $\alpha_\beta_3$ integrin (52). This finding potentially provides a mechanism for cells to generate additional traction at the leading edge of a wound, thereby allowing accelerated migration and wound closure. Interestingly, a member of the 14-3-3 family of adaptor molecules has been reported to bind the cytoplasmic tail of ADAM-22 (which has a non-functional MMP domain), and this complex appears to have a role in co-ordination of $\alpha_\beta_3$ integrin-mediated gloma cell adhesion and spreading on fibronectin (53). It is also conceivable that ADAMs may influence migration independent of metalloproteinase activity. Recently, mutations in the $\alpha_\beta_2$ integrin $\alpha_\beta$ chain of fibrosarcoma cells were found to cause defects in sex myoblast migration and motor axon guidance (54). Many of these mutations were in the disintegrin and the cysteine-rich domains, suggesting that they might be related to integrin function. Subsequent double mutant analyses suggested that UNC-71 probably functions in a combinatorial manner with integrins. In addition, osteoclasts in mice bearing the ADAM-17 catalytic domain deletion were not recruited into the core developing metatarsals, although they still maintained an osteoclast-like phenotype (multinucleated TRAP$^+$ cell (23)).

In summary, this report provides the first evidence that ADAM-17 can act as a ligand for an integrin, $\alpha_\beta_3$, both in trans and cis orientation. The intriguing association of these two molecules during cell migration suggests that further analyses of the functional importance of their binding are warranted.

Acknowledgments—We are grateful to Francisco Sanchez-Madriz, Simon Goodman, and Ken Yamada for gifts of monoclonal antibodies, and to Karl Kader and Philip Woodman for providing cell lines.
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