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ADAM 23/MDC3, a Human Disintegrin That Promotes Cell Adhesion via Interaction with the αvβ3 Integrin through an RGD-independent Mechanism

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ADAM 23 (a disintegrin and metalloproteinase domain)/MDC3 (metalloprotease, disintegrin, and cysteine-rich domain) is a member of the disintegrin family of proteins expressed in fetal and adult brain. In this work we show that the disintegrin-like domain of ADAM 23 produced in *Escherichia coli* and immobilized on culture dishes promotes attachment of different human cells of neural origin, such as neuroblastoma cells (NB100 and SH-S_y5_y) or astrocytoma cells (U373 and U87 MG). Analysis of ADAM 23 binding to integrins revealed a specific interaction with αvβ3, mediated by a short amino acid sequence present in its putative disintegrin loop. This sequence lacks any RGD motif, which is a common structural determinant supporting αvβ3-mediated interactions of diverse proteins, including other disintegrins. αvβ3 also supported adhesion of HeLa cells transfected with a full-length cDNA for ADAM 23, extending the results obtained with the recombinant protein containing the disintegrin domain of ADAM 23. On the basis of these results, we propose that ADAM 23, through its disintegrin-like domain, may function as an adhesion molecule involved in αvβ3-mediated cell interactions occurring in normal and pathological processes, including progression of malignant tumors from neural origin.

INTRODUCTION

Cell–cell and cell–extracellular matrix interactions are essential for the development and maintenance of an organism as well as for the progression of malignant tumors. Likewise, proteolysis of the extracellular matrix is of vital importance for a series of tissue-remodeling processes occurring during both normal and pathological conditions, such as tissue morphogenesis, wound healing, inflammation, and tumor cell invasion and metastasis. These events are mediated by a variety of cell surface adhesion proteins and proteases, with different structural and functional characteristics (Werb, 1997). Among them, a group of recently described proteins called ADAMs (a disintegrin and metalloproteinase domain) have raised considerable interest because of their potential ability to perform both functions, adhesion and proteolysis (Wolfsberg *et al.*, 1995; Blobel, 1997; Wolfsberg and White, 1997). These membrane proteins have a unique domain organization containing pro, metalloproteinase-like, disintegrin-like, cysteine-rich, epidermal growth factor (EGF)-like, transmembrane, and cytoplasmic domains. Some of these domains are similar to those found in a family of soluble snake venom proteins that bind with high affinity to the platelet integrin GPIIb/IIIa, inhibiting platelet aggregation and causing hemorrhage in snake bite victims (Niewiarowski *et al.*, 1994).

ADAMs, also known as cellular disintegrins or MDCs (metalloprotease, disintegrin, and cysteine-rich domains), have been found in a wide variety of mammalian tissues as well as in other eukaryotic organisms, including *Xenopus laevis* (Alfandari *et al.*, 1997; Cai *et al.*, 1998), *Drosophila melanogaster* (Rooke *et al.*, 1996), and *Caenorhabditis elegans* (Podbilewicz, 1996), but not in plants or bacteria. Members of this protein family were first associated with reproductive processes; however, over the last several years the family has widely expanded, and to date, >20 different ADAMs with diverse functions have been identified and characterized at the molecular level. Thus, in addition to a series of family

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Abbreviations used: ADAM, a disintegrin and metalloproteinase domain; CHO, Chinese hamster ovary; EGF, epidermal growth factor; EST, expressed sequence tag; GST, glutathione *S*-transferase; HA, hemagglutinin; MDC, metalloprotease, disintegrin, and cysteine-rich domain; TACE, tumor necrosis factor-α-converting enzyme.

members such as fertilins or cyritestins, involved in spermatogenesis and heterotypic sperm–egg binding and fusion (Blobel *et al.*, 1992; Houliva *et al.*, 1996; Adham *et al.*, 1998), other ADAMs such as meltrin α are implicated in homotypic myoblast–myoblast fusion (Yagami-Hiromasa *et al.*, 1995; Gilpin *et al.*, 1998). Meltrin α and β have also been suggested to play a role in osteoblast differentiation and/or osteoblast activity in bone (Inoue *et al.*, 1998). Furthermore, the cellular disintegrins MS2 (ADAM 8) and decysin have been identified as monocytic and dendritic cell-specific proteins, suggesting that they may be involved in host defense mechanisms (Yoshida *et al.*, 1990; Mueller *et al.*, 1997). Similarly, ADAMTS-1, characterized by the presence of thrombospondin motifs in its amino acid sequence, has been associated with various inflammatory processes (Kuno *et al.*, 1997). Interestingly, ADAMTS-4, another member of this subfamily of disintegrins containing thrombospondin motifs, has been characterized as an aggrecanase responsible for the degradation of cartilage aggrecan in arthritic diseases (Tortorella *et al.*, 1999). Finally, other ADAMs have been found to function as proteolytic enzymes involved in the processing of relevant cellular substrates. In fact, the recently described tumor necrosis factor-α-converting enzyme (TACE) is an ADAM implicated in the release of the proinflammatory membrane-anchored cytokine tumor necrosis factor-α from the plasma membrane (Black *et al.*, 1997; Moss *et al.*, 1997). Similarly, the product of the *kuz* gene from *Drosophila* (ADAM 10) appears to be responsible for the proteolytic activation of the transmembrane protein Notch required for lateral inhibitory signaling during neurogenic differentiation (Pan and Rubin, 1997; Sotillos *et al.*, 1997), although other studies have proposed that Kuz would be required for processing of the Notch ligand Delta (Qi *et al.*, 1999). Finally, Izumi *et al.* (1998) have reported that MDC9/ADAM 9 is involved in the ectodomain shedding of membrane-anchored heparin-binding EGF-like growth factor.

In addition to this variety of physiological functions described for ADAMs, some of these family members have been suggested to play important roles in the development and progression of tumor processes. Thus, ADAM 11 was originally identified as a candidate tumor suppressor gene for human breast cancer (Emi *et al.*, 1993), whereas ADAMTS-1 has been associated with the development of cancer cachexia (Kuno *et al.*, 1997). In addition, several disintegrins have been proposed to be responsible for some pathological features of hematological malignancies such as the premature egression of leukemic cells from bone marrow into the peripheral blood or the generalized connective tissue destruction accompanying these malignant processes (Wu *et al.*, 1997). Furthermore, ADAM 10 is overexpressed in tumors of sympathoadrenal origin such as pheochromocytomas and neuroblastomas (Yavari *et al.*, 1998). Finally, other ADAM family members with proteolytic activity such as TACE have been proposed to play indirect roles in tumor processes through their participation in the proteolytic activation and release of membrane-bound cytokine or growth factor precursors of relevance in cancer (Black *et al.*, 1997; Moss *et al.*, 1997).

These recent findings have stimulated the search for new ADAMs potentially associated with some of the conditions involving cell–cell interactions or extracellular matrix degradation taking place during both normal or pathological conditions (Blobel, 1997; Werb, 1997; Wolfsberg and White, 1997). Recently, we have cloned a full-length cDNA coding for a member of this family that has been called ADAM 23 (GenBank accession number AJ005580) and whose sequence is very similar to that reported for a novel cellular disintegrin (MDC3) recently described by Sagane *et al.* (1998). ADAM 23/MDC3 exhibits the typical structure of ADAM family members and is predominantly expressed in brain, suggesting that it may function as an integrin ligand in cells of neural origin. In this work, we demonstrate that the recombinant disintegrin-like domain of ADAM 23 promotes adhesion of neuroblastoma and astrocytoma cells. We also show that this process is mediated by a specific interaction between $\alpha v\beta 3$ and a short amino acid sequence present in the putative disintegrin loop of ADAM 23. We also provide evidence that $\alpha v\beta 3$ supports adhesion of HeLa cells transfected with a full-length cDNA for ADAM 23. According to these results, we suggest that ADAM 23, through its disintegrin-like domain, may function as an adhesion molecule involved in $\alpha v\beta 3$-mediated cell interactions taking place during normal and pathological processes.

MATERIALS AND METHODS

Materials

Restriction endonucleases and other reagents used for molecular cloning were from Boehringer Mannheim (Mannheim, Germany). Double-stranded DNA probes were radiolabeled with [α-^{32}P]dCTP (3000 Ci/mmol) purchased from Amersham International (Buckinghamshire, United Kingdom) using a commercial random-priming kit from the same company. A human brain cDNA library constructed in λDR2 and Northern blots containing polyadenylated RNAs from different adult and fetal human tissues were from Clontech (Palo Alto, CA). Synthetic peptides were obtained from the Molecular Biology Facilities Unit (University of Leicester, Leicester, United Kingdom). NB100 and SH-S_y5_y human neuroblastoma cells were kindly provided by Dr. F. Sánchez-Madrid (Hospital de la Princesa, Madrid, Spain), and Drs. F. Barros and T. Giráldez (Universidad de Oviedo). U373 and U87 MG astrocytoma cell lines were provided by Dr. A. Nakano (Hyogo College of Medicine, Hyogo, Japan) All media and supplements for cell culture were obtained from Sigma (St. Louis, MO) except for fetal calf serum, which was from Boehringer Mannheim.

Isolation of a cDNA Clone for ADAM 23 from a Human Brain cDNA Library

A search of the GenBank database of human expressed sequence tags (ESTs) for sequences with homology to members of the ADAM family led us to identify a sequence (R52569; Washington University-Merck EST Project, St. Louis, MO) derived from a brain cDNA clone and showing significant similarity to sequences of previously described ADAMs. To obtain this DNA fragment, we performed PCR amplification of a human brain cDNA (Clontech) with two specific primers, 5′-CAACAAAGCTATTTGAGCCCACGG and 5′-TTGGTGGGCACTGACCAGAGTCT, derived from the R52569 sequence. The PCR reaction was carried out in a GeneAmp 2400 PCR system from Perkin Elmer (Norwalk, CT) for 40 cycles of denaturation (94°C, 15 s), annealing (64°C, 20 s), and extension (72°C, 20 s). The 262-bp PCR product amplified from human brain cDNA was cloned into a *Sma*I-cut pBluescript II SK vector (Stratagene, La Jolla, CA), and its identity was confirmed by nucleotide sequencing. This cDNA was then excised from the vector, radiolabeled, and used to screen a human brain cDNA library according to standard procedures (Maniatis *et al.*, 1982).

Northern Blot Analysis

Northern blots containing poly(A)$^+$ RNAs from different fetal and adult human tissues were obtained from Clontech. These blots were prehybridized at 42°C for 3 h in 50% formamide, 5× SSPE, 2× Denhardt's solution, 0.1% SDS, and 100 μg/ml denatured herring sperm DNA and then hybridizated for 16 h under the same conditions with the full-length cDNA isolated for ADAM 23. Filters were washed with 0.2× SSC and 0.1% SDS for 2 h at 50°C and subjected to autoradiography. RNA integrity and equal loading were assessed by hybridization with an actin probe as indicated by Clontech.

Reverse Transcription-PCR Amplification

To assay the presence of ADAM 23 in neuroblastoma cell lines, total RNA was isolated from NB100 and SH-S$_y$5$_y$ cells by guanidium thiocyanate-phenol-chloroform extraction and used for cDNA synthesis with the RNA PCR kit from Perkin Elmer. After reverse transcription using 1 μg of total RNA and random hexamers as primer according to the instructions of the manufacturer, the whole mixture was used for PCR with the two specific oligonucleotides corresponding to the disintegrin domain of ADAM 23 as described above. Negative controls were performed using all reagents with the exception of random hexamers.

Construction of an Expression Vector for ADAM 23 and Expression in Escherichia coli

A 975-bp fragment of the ADAM 23 cDNA containing the disintegrin-like domain was generated by PCR amplification with primers 5′-TAGGGATCCCAAAGCTATTTGAGCCCA and 5′-ATGAAGATTTGGTGGGCA. The PCR amplification was performed for 20 cycles of denaturation (95°C, 20 s), annealing (52°C, 20 s), and extension (68°C, 20 s), followed by 10 additional cycles of denaturation (95°C, 15 s), annealing (62°C, 15 s), and extension (68°C, 2 min) using the Expand Long PCR kit and the GeneAmp 9700 PCR system. Because of the design of the oligonucleotides, the amplified fragment could be cleaved at the 5′ end with *Hin*dIII and ligated in frame into the pGEX-3x *E. coli* expression vector (Invitrogen, San Diego, CA) previously cleaved with *Hin*dIII–*Sma*I. The expression vector was transformed into BL21(DE3)pLysS competent *E. coli* cells and grown on agar plates containing chloramphenicol and ampicillin. Single colonies were used to inoculate 2-ml cultures in 2YT medium supplemented with 33 μg/ml chloramphenicol and 50 μg/ml ampicillin. Five hundred microliters of the corresponding culture were used to inoculate 200 ml of 2YT medium containing the above antibiotics. After culture reached an OD_{600} of 0.6, expression was induced by addition of isopropyl-1-thio-β-D-galactopyranoside (0.5 mM final concentration) followed by further incubation for 3–20 h at 30°C. Cells were collected by centrifugation, washed, and resuspended in 0.05 vol of PBS, lysed by using a French press, and centrifuged at 20,000 × *g* for 20 min at 4°C. The soluble extract was incubated with glutathione-Sepharose 4B (Pharmacia, Uppsala, Sweden) and eluted with glutathione elution buffer (10 mM reduced glutathione in 50 mM Tris-HCl, pH 8.0) following the manufacturer's instructions.

Adhesion Assays

Cell adhesion assays were essentially performed as previously described by Luque *et al.* (1994). Briefly, 96-well immunoplates (MaxiSorp; Nunc, Roskilde, Denmark) were coated with 0.1 ml of PBS containing different amounts of BSA, glutathione *S*-transferase (GST), and ADAM 23/GST. After incubating for 16 h at 4°C, wells were blocked with Dulbecco's modified Eagle's medium containing 2.5% BSA for 2 h at 37°C. Then, NB100 neuroblastoma cells (~50,000 cells per well) were added in Dulbecco's modified Eagle's medium supplemented with 1% BSA and incubated at 37°C for 2 h. For experiments directed to analyze the effect of divalent cations, the cells were washed three times in PBS and resuspended in the same buffer supplemented with either 1 mM $MgCl_2$, 50 μM $MnCl_2$, and 1 mM $CaCl_2$ or 1 mM $MgCl_2$ plus 5 mM EDTA. Nonbound cells were removed by rinsing the wells with serum-free medium, whereas bound cells were fixed with methanol and stained with Giemsa. Cells were counted per unit area with the aid of an inverted light microscope, using a 20× high-power objective and an ocular grid. For inhibition studies cells were pretreated for 30 min before the addition to the coated wells of mAb LM 609 (used at 1:400 dilution of ascites) or synthetic peptides (20 or 40 μg/ml) corresponding to the disintegrin loop of ADAM 23 (AVNEDCDIT, peptide 330) or a "scrambled" peptide (DCVTNIAE, peptide 331). In all cases, experimental treatments were performed in triplicate with a minimum of three areas counted per well.

Scanning Electron Microscopy

Glass coverslips (12-mm diameter) were immersed in 60% HNO_3 for 1 h, washed with distilled water, immersed in 7% NaOH, and washed with water again. After drying, coverslips were placed in a 24-well tissue culture plate and coated with ADAM 23 or fibronectin in PBS (20 μg/ml). After overnight incubation at 4°C, coverslips were washed with PBS to remove free protein and coated with 2.5% BSA. NB100 cells were then seeded (~15,000 cells per cm^2) in the same buffer used for cell adhesion experiments and allowed to adhere for 2 h at 37°C. Unbound cells were then removed by washing with free serum medium, and adherent cells were fixed with 2.5 glutaraldehyde in 0.1 M cacodylate buffer, pH 7.5, for 3 h and then washed, osmicated, dehydrated with acetone, critical point dried, and gold coated. Cells were then viewed under a JEOL (Tokyo, Japan) JSM 6100 scanning electron microscope and photographed.

Immunofluorescence Microscopy

NB100 cells were grown on glass coverslips as described above, fixed with 3.7% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized with 0.2% Triton X-100 for 10 min. Coverslips were then incubated with 10% fetal bovine serum in PBS (30 min), followed by a 1:400 dilution of a commercial anti-vinculin mAb (Sigma) for 1 h. After washing with PBS, incubation was made with a mix of a 1:500 dilution of a goat anti-rabbit immunoglobulin G FITC-conjugated antibody (Amersham). For staining of filamentous actin, 0.1 μg/ml rhodamine-phalloidine was included during incubation with the secondary antibodies. Finally, washed coverslips were mounted, and cells were examined using a Zeiss (Thornwood, NY) fluorescent microscope equipped with a charge-coupled device camera (Photometrics, Tucson, AZ).

Generation of Chinese Hamster Ovary (CHO) Cells Expressing Different Human Integrins

The development of these cell lines has been described previously (Zhang *et al.*, 1998). Briefly, cDNA constructs for different integrins were transfected into CHO cells, together with a neomycin resistance gene. After selection with G-418, cells stably expressing human integrins were cloned by sorting to obtain high expressers. The α2, α3, α4, α5, α6, and αv subunits of human integrins were expressed in combination with the β1 subunit of hamster, whereas β subunits were expressed in combination with α human subunits. The β3-CHO cells expressed human β3/hamster αv hybrid.

Construction of Eukaryotic Expression Vectors for ADAM 23-HA and Immunolocalization

A full-length cDNA encoding ADAM 23 was PCR amplified with oligonucleotides Ad23-D (5′-TATGAGCCATGAAGCCGCCCG-3′) and Ad23-R (5′-GATGGGGCCTTGCTGAGTAGG-3′) and cloned in the *Eco*RV site of a modified pcDNA3 vector containing a 24-bp linker coding for the hemagglutinin (HA) epitope of human influ-

enza virus. Thus, the resulting ADAM 23 protein was HA tagged at the COOH terminus. HeLa cells were transfected with 1 μg of plasmid pcDNA3-ADAM 23-HA or pcDNA alone using LipofectAMINE reagent (Life Technologies, Gaithersburg, MD), according to the manufacturer's instructions. Transfected cells were used for binding experiments to purified $\alpha v\beta 3$ or to protein extracts from integrin-transfected CHO cells as above, with the exception that experiments were performed without divalent cations. For immunolocalization experiments, 48 h after transfection, cells were fixed for 10 min in cold 4% paraformaldehyde in PBS, washed in PBS, and incubated for 10 min in 0.2% Triton X-100 in PBS. Fluorescent detection was performed by incubating the slides with mAb 12CA5 (Boehringer Mannheim) against HA (diluted 1:100), followed by another incubation with goat anti-mouse fluoresceinated antibody (diluted 1:50). Antibodies were diluted in blockage solution (15% FCS in PBS). After washing in PBS, slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA) and observed in a Bio-Rad (Hercules, CA) confocal laser microscope.

Site-directed Mutagenesis

The E466A mutation in the disintegrin loop of ADAM 23 was carried out by PCR-based methods. An oligonucleotide containing the mutation 5′-GTAATATCACACGCGTTCACAGCA (with G indicating a change of T to G in the original sequence) and a second oligonucleotide containing a *Bam*HI site (5′-GTGGATCCCCAAGCTATTG) were first used to PCR amplify a DNA fragment. This amplified product was then used as a "megaprimer" for a second PCR amplification with an oligonucleotide corresponding to the 3′ end of the cloning site of pGEX-3X. PCR conditions were 94°C, 2 min (1 cycle), and 94°C, 0.1 s; 60°C, 0.1 s; and 68°C, 30 s (20 cycles). The PCR product of the expected size was digested with *Bam*HI and *Eco*RI and cloned in pGEX-3X. The presence of the mutation was confirmed by nucleotide sequencing. Finally, production of the recombinant mutant protein in *E. coli* was carried out as described above.

Western Blot Analysis

Purified integrins ($\alpha v\beta 3$, $\alpha 1\beta 1$, or $\alpha 5\beta 1$, 0.3 μg each; Chemicon International, Temecula, CA) were incubated with Sepharose 4B beads containing 0.5 μg of disintegrin-GST, in a buffer containing 50 mM Tris-HCl, 200 mM NaCl, and 0.2 mM $MnCl_2$, pH 7.4, for 4 h at 37°C. After incubation, beads were washed six times with 200 μl of the same buffer to remove unbound protein. Beads were then resuspended in Laemmli buffer, and after boiling, solubilized proteins were loaded in a 6% SDS-PAGE gel and visualized by silver staining. Alternatively, samples were blotted to a nitrocellulose membrane, and the presence of αv or β3 integrin subunits was detected using polyclonal antibodies raised against these subunits (kindly provided by Drs. R. Lacalle and C. Martínez-A., Centro Nacional de Biotecnología, Madrid, Spain). Similarly, the putative presence of β1 integrin subunits was examined with the B3B11 mAb (mAb 2251; Chemicon). Western-blots were visualized by enhanced chemiluminescence according to the manufacturer's instructions (Amersham).

RESULTS

Cloning of a Full-Length cDNA Encoding ADAM 23/MDC3, a Member of the Cellular Disintegrin Family

To identify novel members of the ADAM family produced by human tissues, we screened the GenBank database of ESTs looking for sequences with similarities to those of previously described family members. This analysis allowed us to identify a 405-bp EST (R52569) that, when translated, exhibited significant amino acid sequence similarity to the disintegrin domain characteristic of ADAMs. A cDNA containing part of this EST was generated by PCR amplification of DNA prepared from a human brain cDNA library and used as a probe to screen this library. Sequence analysis of one of the positive clones (called 6D) revealed an open reading frame coding for a protein of 832 amino acids with a predicted molecular mass of 91.9 kDa (European Molecular Biology Laboratory accession number AJ005580). An alignment of the deduced amino acid sequence revealed that this protein possesses all characteristic domains of the ADAM family members, including propeptide, metalloproteinase-like, disintegrin-like, and cysteine-rich domains, an EGF-like repeat, a transmembrane domain, and a cytoplasmic tail (Figure 1). Further analysis of the identified amino acid sequence revealed that it was virtually identical to that derived from a brain cDNA cloned by Sagane *et al.* (1998) during preparation of this manuscript and called MDC3. Both sequences are identical in the coding region, although the cDNA isolated in this work is ~1 kb longer than that of MDC3 and shows some sequence discrepancies, which could be derived from genetic polymorphisms or sequencing errors. Following the nomenclature system for cellular disintegrins (see http://www.med.virginia.edu/~jag6n/whitelab.html), we would assign the name ADAM 23 to this enzyme. Further comparative analysis of the ADAM 23 amino acid sequence revealed a significant similarity with other human ADAMs, the maximum percentage of identities being with ADAM 11 (53%) and ADAM 22 (51%). Expression analysis of ADAM 23 in human tissues revealed a restricted pattern of expression to fetal and adult brain (Figure 2A; also see Sagane *et al.*, 1998). It is also remarkable that tumor cells from neural origin such as NB 100, SH-S_y5_y, U373, and U87 MG, also expressed this gene. In contrast, a variety of tumor cell lines from diverse sources such as HL-60 (promyelocytic leukemia), K-562 (chronic myelogenous leukemia), Raji (Burkitt's lymphoma), HeLa (cervical adenocarcinoma), SW480 (colorectal adenocarcinoma), and A549 (lung adenocarcinoma) did not show significant levels of ADAM 23 expression (Figure 2B; our unpublished observations).

Production of Recombinant ADAM 23 in Bacterial Cells and Analysis of its Cell Adhesive Properties

According to the above structural data, ADAM 23 has a number of features characteristic of ADAM family members. However, its deduced amino acid sequence lacks essential residues conserved in metalloproteinases (Figure 1), suggesting that this protein could be involved in cell adhesion processes rather than in protease-mediated events. As a preliminary step to elucidate whether ADAM 23 is active in cell–cell adhesion processes, we expressed its disintegrin-like domain in a bacterial system following the strategy described for expression of other cellular disintegrins (Zhang *et al.*, 1998). The predicted disintegrin domain of ADAM 23 was subcloned into the expression vector pGEX-3X, and the resulting plasmid, called pGEX-3X ADAM 23, as well as the original vector, were transformed into *E. coli* BL21(DE3)pLysS. Transformed bacteria were induced with isopropyl-1-thio-β-D-galactopyranoside, and protein extracts were analyzed by SDS-PAGE. According to the obtained results, extracts from bacteria transformed with the recombinant plasmid contained a fusion protein of ~40 kDa,

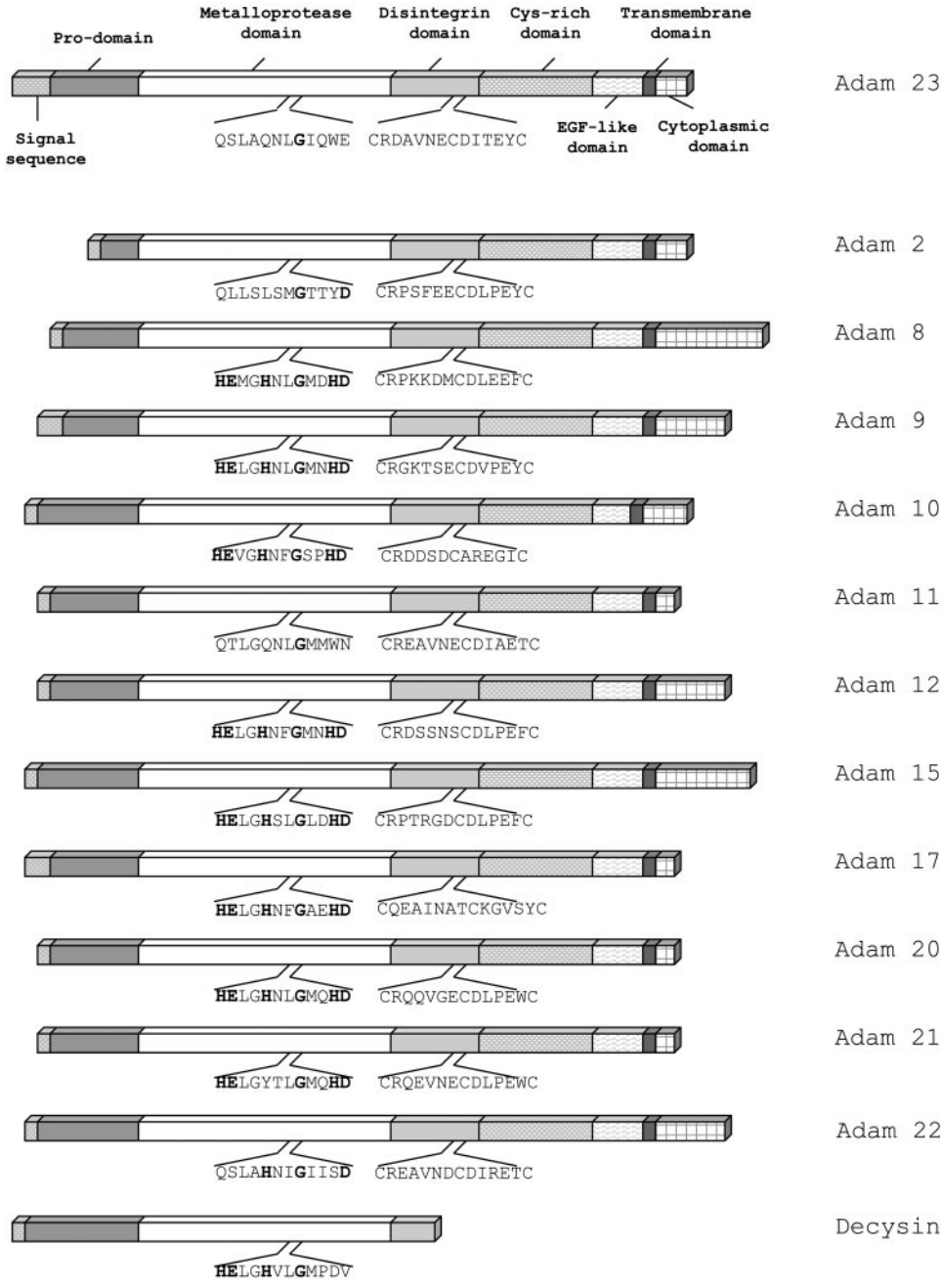
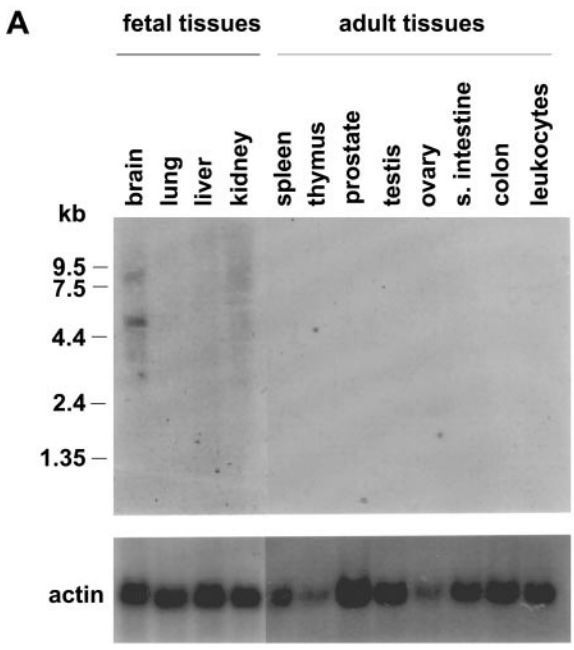


Figure 1. Comparison of the domain structure of ADAM 23 and human ADAMs. The amino acid sequences of the regions around the consensus sequence for metalloproteinases (**HE**XX**H**XX**G**XX**H**) and the putative integrin-binding loops of ADAMs are shown. Sequences for human fertilin α (ADAM 1) and cyritestin 1 (ADAM 3) are not included, because their respective genes are not functional (Jury *et al.*, 1997; Frayne and Hall, 1998). The disintegrin domain of decysin is truncated and lacks the region compared in the alignment of the remaining family members. All amino acid sequences were extracted from the SwissProt database. The modular structure of members of the recently described ADAM-TS subfamily containing thrombospondin-1 repeats is shown by Hurskainen *et al.* (1999).

which was not present in the control extracts (Figure 3). The recombinant protein was purified by affinity chromatography in a glutathione-Sepharose 4B column, which was eluted with a reduced glutathione-containing buffer. After elution and SDS-PAGE analysis of proteins present in the chromatographic eluate, a single band of the expected size was detected (Figure 3).

To examine the activity of the purified disintegrin domain of ADAM 23, wells of microtiter plates were coated with the recombinant protein and seeded with NB100 human neuroblastoma cells. After rinsing the wells to remove unbound cells, bound cells were stained and quantified. As shown in Figure 4B, ADAM 23-GST promoted cell adhesion in a manner similar to that observed when wells were coated with fibronectin (Figure 4A). By contrast, wells coated with GST, albumin, or buffer alone did not support any significant cell adhesion. Morphological studies of NB100 cells adherent to ADAM 23-GST or fibronectin by using light and scanning electron microscopy revealed differences in cell morphology that were mainly related to changes in the number and length of surface protrusions (Figure 4, C and D). We also examined



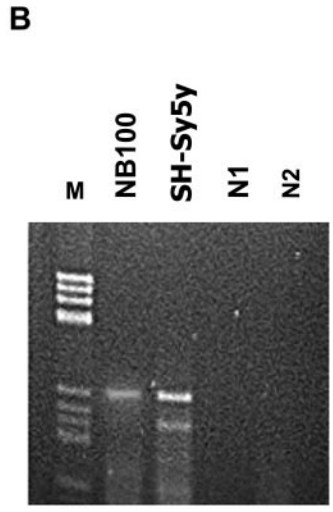


Figure 2. Analysis of ADAM 23 expression in human tissues and cell lines. (A) Approximately 2 μg of polyadenylated RNA from the indicated tissues were analyzed by hybridization with the full-length cDNA isolated for human ADAM 23. The positions of RNA size markers are shown. Filters were subsequently hybridized with a human actin probe to ascertain the differences in RNA loading among the different samples. (B) Reverse transcription-PCR analysis of ADAM 23 expression in human neuroblastoma NB100 and SH-S_y5_y cells. N1 and N2 indicate negative controls for each cell line. M, molecular size markers.

the structure of the actin cytoskeleton in NB100 cells adherent to either ADAM 23 or fibronectin (Figure 4, E–H). Neuroblastoma cells adherent to fibronectin showed a conventional F-actin distribution including relatively little F-actin in the central region of the cell and concentrated F-actin in a layer just beneath the plasma membrane (Figure 4E). Cells adherent to ADAM 23 contained actin filaments mainly located at specific cortical regions, but, compared with cells adherent to fibronectin, they tended to have decreased levels of assembled actin filaments and a lower polarized pattern (Figure 4F). In both cells adherent to ADAM 23 and cells adherent to fibronectin, phalloidine labeling was not uniform but usually was relatively dense in some areas and relatively sparse in others. Some of the dense labeling occurred in fairly distinct patches localized in close apposition to the plasma membrane. To confirm that these patches were actin–filament attachment sites in the plasma membrane and to study their distribution, staining of the same cells with antibodies to vinculin was performed. A clear relationship among the sites of vinculin localization, the actin–filament bundles, and the sites of filopodial protrusion was observed (Figure 4, G and H). Although differences in the vinculin labeling pattern between cells adherent to either ADAM 23 or fibronectin were found, such differences were restricted to the degree of aggregation, being higher in cells adherent to fibronectin (Figure 4, G and H). Nevertheless, in both cases, vinculin-positive patches were heterogeneously distributed, being concentrated at specific cortical regions, which presumably corresponded to the leading edge of the cells.

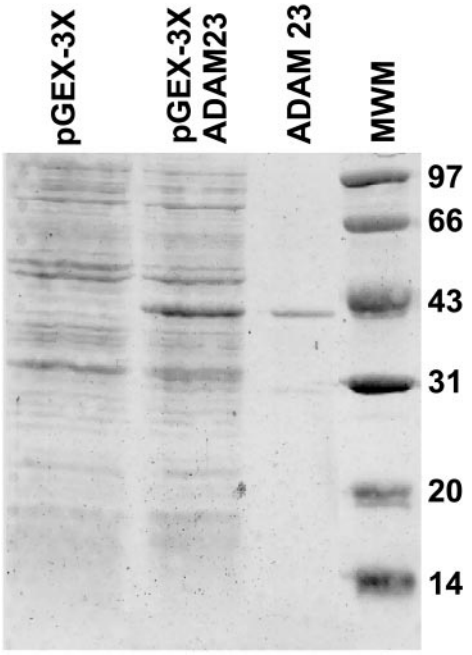


Figure 3. SDS-PAGE analysis of purified recombinant ADAM 23 produced in *E. coli*. Five-microliter aliquots of bacterial extracts (pGEX-3X and pGEX-3X ADAM 23) as well as 1 μl of purified ADAM 23 were analyzed by SDS-PAGE. The sizes in kilodaltons of the molecular size markers (MWM) are indicated at the right.

Further analysis of the ADAM 23-promoted cell adhesiveness revealed that this effect was dose dependent (Figure 5). In addition, the attachment of NB 100 neuroblastoma cells was stimulated in the presence of divalent cations such as Mn^{2+} and Mg^{2+} (Figure 5). Similar results were obtained when these experiments were performed with other cells from neural origin such as SH-S_y5_y, U373, and U87 MG. In contrast, when similar experiments were performed with other cell lines from different sources,

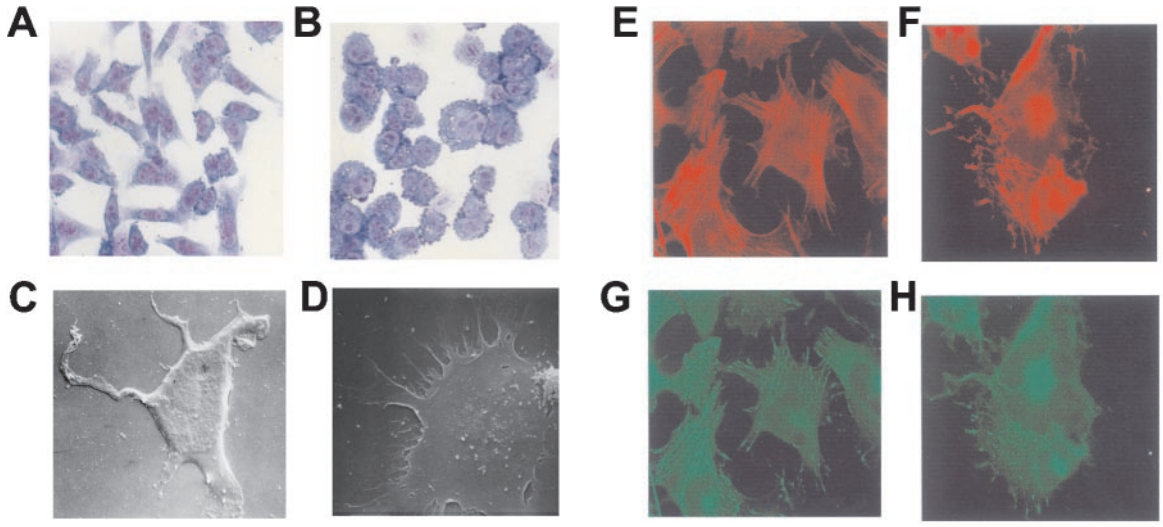


Figure 4. Analysis of the role of ADAM 23 in neuroblastoma cell adhesion. Adhesion of NB 100 cells to dishes coated with 10 μg/ml fibronectin (A) or with 10 μg/ml recombinant ADAM 23 (B) is shown. Several differences in cell morphology are found at the light microscopic level. Scanning electron microscopy proved that differences in cell morphology between NB 100 cells adherent to fibronectin (C) and those adherent to ADAM 23 (D) are mainly due to variations in the number and length of surface protrusions. Cells adherent to fibronectin (C) have a relatively low number of long asymmetric filopodial extensions, whereas those attached to ADAM 23 are devoid of long surface extensions but show many short protrusions resembling microspikes, most of which appeared to be firmly attached to the glass coverslip. The effect of ADAM 23 on F-actin and vinculin distribution is shown in E–H. Confocal optical sections of NB100 cells adherent to fibronectin (E and G) and ADAM 23 (F and H) were double labeled for F-actin (rhodamine-phalloidine; red) and vinculin (anti-vinculin, visualized with FITC-labeled goat anti-rabbit; green). Neuroblastoma cells adherent to fibronectin (E) show relatively little F-actin in the central region of the cell and numerous parallel bundles at the axis of the filopodial protrusions. In cells adherent to ADAM 23 (F), the assembly of actin filaments is lower, but they are mainly located at specific cortical regions and display a moderate polarization. Vinculin in neuroblastoma cells adherent to fibronectin (G) is mainly located at the sites of filopodial protrusion. Vinculin-positive patches in cells adherent to ADAM 23 (H) show a lower degree of aggregation, but they are also concentrated at specific cortical regions.

including HT1080, HeLa, and T47D cells, no significant ADAM 23-mediated adhesion was observed (our unpublished observations). These results suggest that the effect of this cellular disintegrin on cell adhesion may be dependent on the presence of specific integrins in the adherent cells.

ADAM 23 Promotes Cell Adhesion via Interaction with αvβ3 Integrin

To evaluate the possibility that ADAM 23 mediates cell adhesion through interaction with specific integrins, we used a panel of CHO cells expressing different recombinant integrins. According to previous data (Takada *et al.*, 1992; Takagi *et al.*, 1997), parent CHO cells express α5β1 as a major integrin but do not express significant amounts of β2 or β3 integrins. However, when wells of microtiter plates were coated with the disintegrin domain of ADAM 23 (10 μg/ml) and seeded with these parent CHO cells, no significant cell adhesion-promoting effect was observed (Figure 6A). Similar results were obtained with CHO cells expressing a variety of exogenous integrins, including α2β1, α3β1, α4β1, α6β1, and αvβ1. In contrast, the recombinant ADAM 23 promoted adhesion of β3-CHO cells that express functional αvβ3 (Figure 6A). These results are consistent with the proposal that ADAM 23 mediates cell adhesion through interactions with αvβ3. To provide further evidence on this question, we examined the effect of a function-blocking anti-αvβ3 mAb (LM 609) on the cell adhesion properties supported by ADAM 23. As shown in Figure 6B, adhesion of ADAM 23 to β3-CHO cells was substantially diminished in the presence of LM 609. Similarly, this anti-αvβ3 antibody was able to reduce the ADAM 23-mediated adhesion of NB100 neuroblastoma cells, whereas a β1-blocking antibody (LIA 1/2) did not show any significant effect on this activity (Figure 6B). The specificities of function-blocking antibodies LM 609 and LIA 1/2 have been described previously (Cheresh and Spiro, 1987; Luque *et al.*, 1994).

To further examine αvβ3–ADAM 23 interaction, Sepharose beads containing the ADAM 23 disintegrin domain fused to GST were incubated with purified αvβ3 integrin. After extensive washing to remove the unbound integrin, the presence of bound αvβ3 was examined by SDS-PAGE of proteins solubilized in an SDS-containing buffer. As shown in Figure 7A, two bands corresponding to αv (145 kDa) and β3 (95 kDa) were detected in extracts from beads containing ADAM 23-GST but not in those derived from beads containing GST alone. The identity of these bands as αv and β3 was confirmed by Western blot analysis with antibodies raised against each integrin subunit (Figure 7B). It is remarkable that similar experiments performed with protein extracts from other integrin-transfected CHO cells or with other purified integrins such as α1β1 and α5β1 did not reveal any

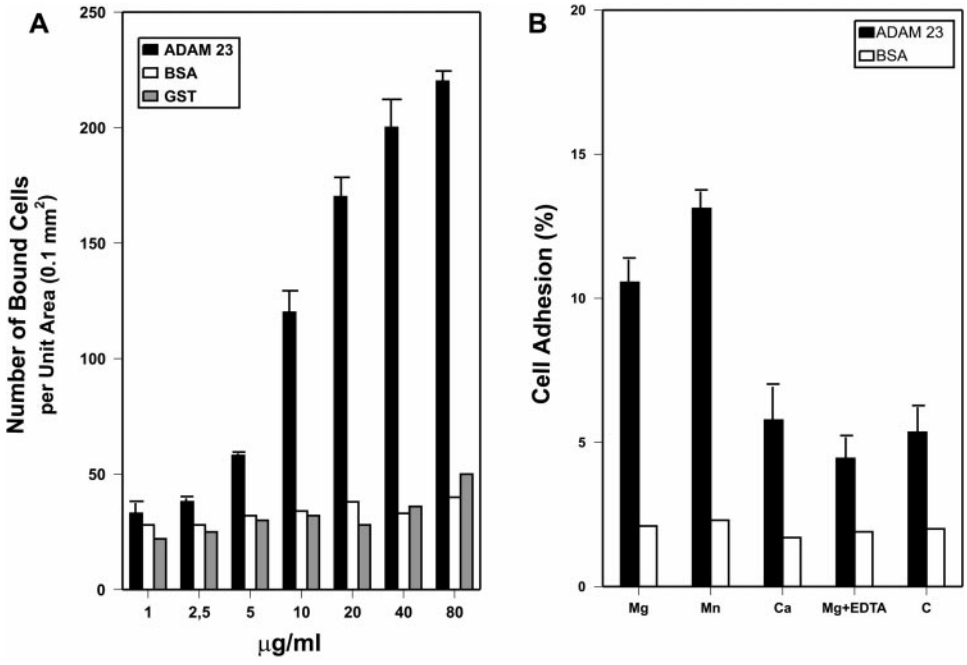


Figure 5. Dose- and cation-dependent analysis of the effect of ADAM 23 on NB100 adhesion. (A) Culture dishes were coated with the indicated concentrations of ADAM 23, BSA, and GST and blocked for nonspecific binding with BSA (2.5%). Cells were allowed to adhere for 2 h at 37°C. Nonbound cells were removed by washing, and adhesion was determined by counting bound cells per unit area using a 20× high-power objective and an ocular grid. (B) Analysis of the effect of divalent cations on NB100 adhesion mediated by ADAM 23. The experiments were performed as above with the exception that cells were washed three times in PBS and resuspended in the same buffer supplemented either with 1 mM $MgCl_2$, 50 μM $MnCl_2$, and 1 mM $CaCl_2$ or 1 mM $MgCl_2$ plus 5 mM EDTA. C, control cells, corresponding to NB100 cells plated on wells coated with BSA in the absence of divalent cations. The results presented are the mean of three independent experiments.

evidence of interaction with the recombinant ADAM 23 (Figure 7B). This result provides additional evidence of the specificity of the interaction between $\alpha v\beta 3$ integrin and the ADAM 23 disintegrin domain.

Finally, we performed an additional series of experiments directed to analyze the interaction between $\alpha v\beta 3$ and ADAM 23 in the context of the full-length ADAM 23 protein. To this purpose, the full-length cDNA for ADAM 23, containing a linker encoding the HA epitope at its 3′-end, was cloned into the eukaryotic expression vector pcDNA3. The resulting plasmid (pcDNA3-ADAM 23-HA) was transfected into HeLa cells, and then the ability of transfected cells to bind $\alpha v\beta 3$ was examined. As shown in Figure 8A, wells of microtiter plates coated with this integrin strongly supported cell adhesion of HeLa cells transfected with the ADAM 23 expression vector. In contrast, HeLa cells transfected with pcDNA3 alone did not support any significant cell adhesion (Figure 8A). To provide additional evidence that ADAM 23 was located at the cell surface, a prerequisite for mediating the observed cell adhesion effect, HeLa cells transfected with pcDNA3-ADAM 23-HA were analyzed by immunofluorescence with an mAb against the HA viral epitope. As shown in Figure 8B, a clear fluorescent pattern surrounding transfected cells was visualized in a serial op-

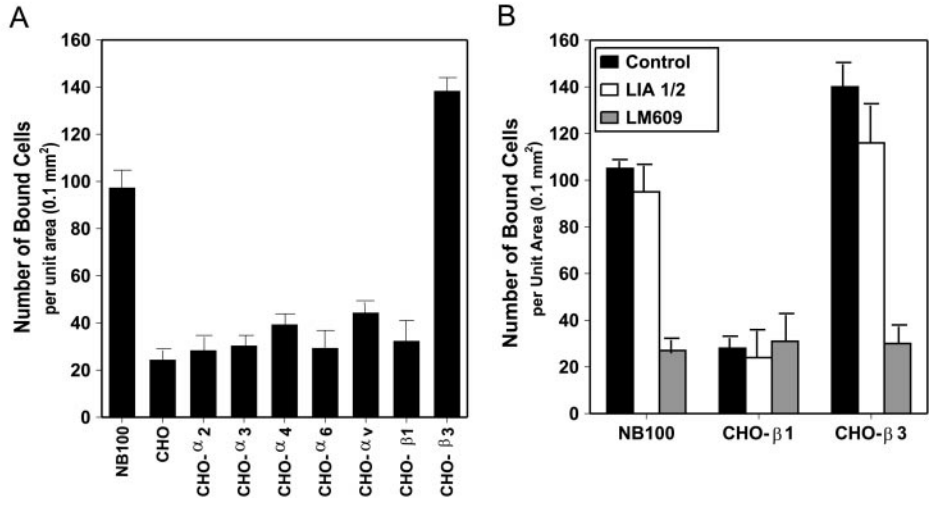


Figure 6. Analysis of ADAM 23 interaction with cell lines expressing specific integrins. (A) Microtiter plates were coated with ADAM 23-GST (10 μg/ml) and seeded with parental CHO cells or with CHO cells transfected with the indicated integrins. (B) Effect of a function-blocking anti-$\alpha v\beta 3$ antibody (LM 609) on $\beta 3$-CHO and NB100 neuroblastoma cell adhesion mediated by ADAM 23. Control, control sample (without addition of antibody); LIA 1/2, samples analyzed with this blocking anti-$\beta 1$ antibody.

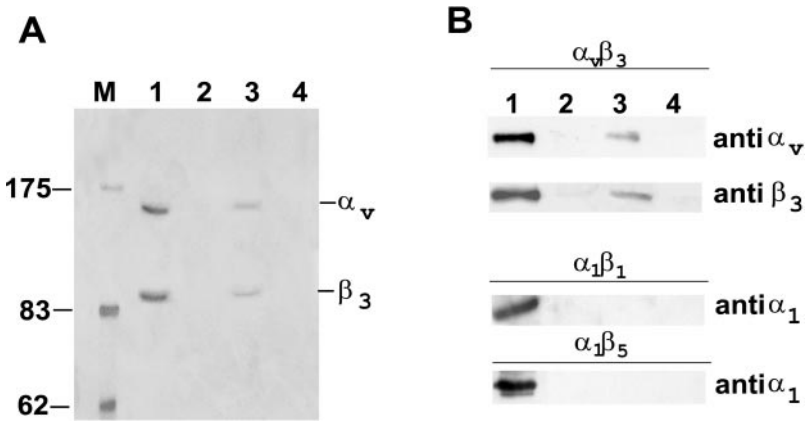


Figure 7. Interaction between human αvβ3 and the disintegrin domain of ADAM 23. (A) Purified αvβ3 integrin and Sepharose beads containing recombinant ADAM 23-GST were incubated in a Mn-containing buffer. After repeated washings, the presence of bound αvβ3 was analyzed by SDS-PAGE of proteins solubilized from Sepharose beads after treatment with Laemmli denaturing buffer. Staining was carried out with silver nitrate. (B) Western blot analysis of the solubilized material from Sepharose beads using antibodies anti-αv, anti-β3, and anti-β1. M, molecular weight markers (indicated at the left). Lane 1, 300 ng of αvβ3, α1β1, or α5β1; lane 2, beads incubated with the indicated integrins; lane 3, beads containing recombinant ADAM 23-GST (500 ng) incubated with the integrins; lane 4, beads containing GST incubated with the indicated integrins.

tical section obtained by the confocal microscope. In contrast, untransfected HeLa cells did not show any evidence of immunofluorescence signal at the cell surface. Taken together, these results are consistent with the proposal that ADAM 23 located at the cell surface is able to promote αvβ3-mediated cell adhesion.

Interaction between ADAM 23 and αvβ3 is Mediated by a Short Amino Acid Sequence Present in the Disintegrin-like Domain of ADAM 23

Analysis of the amino acid sequence of ADAM 23 shows the absence of any RGD motif (Figure 1; European Molecular Biology Laboratory accession number AJ005580). This sequence has been found to be the major structural determinant supporting αvβ3-mediated interactions in different systems, including those involving metargidin, the only cellular disintegrin described to date containing an RGD motif (Krätzschmar *et al.*, 1996; Herren *et al.*, 1997; Zhang *et al.*, 1998). A comparison of the amino acid sequence of different human disintegrins around the putative region involved in integrin binding allowed us to select a short motif (AVNECDIT) as a candidate to mediate the above-observed effect of ADAM 23 on cell adhesion (Figure 1). To determine whether this sequence is actually involved in the adhesive effect, we first mutated the Glu residue of the central position to Ala. The disintegrin-like domain of the mutant protein, designated mutADAM 23, was expressed as a fusion protein with GST following the same strategy described above for the wild-type disintegrin domain of ADAM 23. After affinity chromatography purification (Figure 9A, inset), the recombinant mutant protein was used for cell adhesion assays. As shown in Figure 9A, the mutant ADAM 23 showed a significantly lower adhesion-promoting activity of NB100 cells than the effect observed when the wild-type ADAM 23 protein was used. Similarly, when wells of microtiter

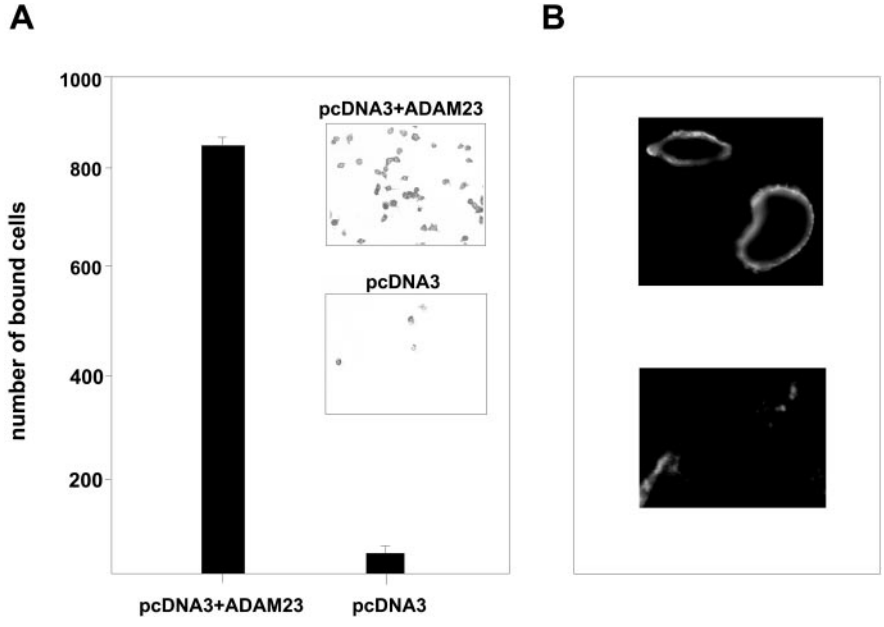


Figure 8. Interaction between human αvβ3 and HeLa cells transfected with full-length cDNA for ADAM 23. (A) HeLa cells were transfected with the expression vector pcDNA3-ADAM 23-HA containing the full-length cDNA for ADAM 23 linked to a 24-bp oligonucleotide coding for the HA epitope. Transfected cells were added to microtiter plates coated with 10 μg/ml purified αvβ3. Cells were allowed to adhere for 2 h at 37°C. Nonbound cells were removed by washing, and adhesion was observed at the light microscopic level using a 20× high-power objective. (B) Immunofluorescence analysis of HeLa cells transfected with plasmid pcDNA3-ADAM 23-HA. Top, transfected cells were incubated with a monoclonal anti-HA antibody (12CA5) diluted 1:2500 followed by another incubation with goat anti-mouse fluoresceinated antibody (diluted 1:50). Fluorescence was observed under a confocal laser microscope and localized to the surface of the ADAM 23-HA transfected cells. Bottom, negative control showing HeLa cells transfected with pcDNA3.

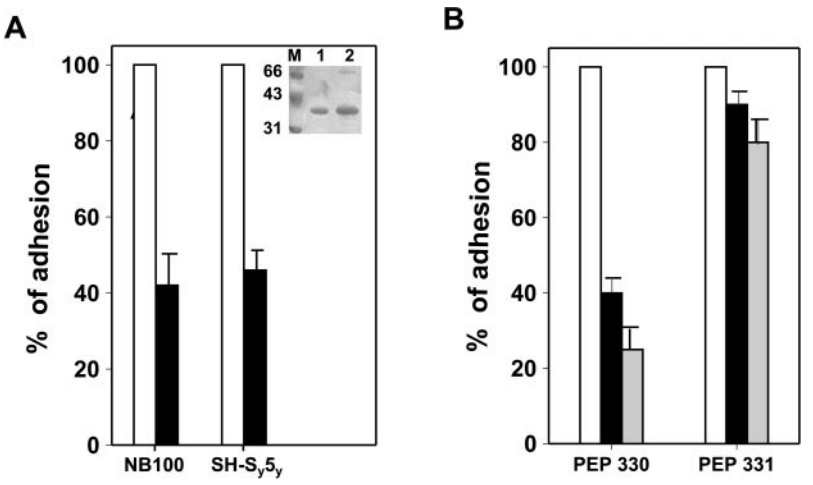


Figure 9. Delineation of the ADAM 23 region mediating the interaction with $\alpha v\beta 3$. (A) Effect of a point mutation (E→A) in the sequence of ADAM 23 on its adhesive-promoting properties represented as percentage of adhesion using neuroblastoma cell lines. ADAM 23 wild-type and experiments using mutant ADAM 23 are shown as white and black bars, respectively. Inset, SDS-PAGE gel with both ADAM 23 wild-type (lane 1) and E→A mutant (lane 2). M, molecular weight markers. (B) Effect of a synthetic peptide (AVNECDIT, pep 330) on the adhesion of NB100 cells. Pep 331 indicates the assay carried out with a scrambled peptide (DCVTNIAE). Two different concentrations of peptides were used: 20 μg/ml (black bars) and 40 μg/ml (gray bars). White bars indicate control using NB100 cells grown on ADAM 23.

plates were coated with the mutant ADAM 23 and seeded with SH-S$_y$5$_y$ neuroblastoma cells, the observed cell adhesion-promoting effect was ~40% compared with that obtained with the wild-type protein (Figure 9A).

To further examine the role of the sequence motif AVNECDIT in mediating the cell adhesion-promoting properties of ADAM 23, we next prepared a synthetic peptide enclosing this region (pep330) and a scrambled peptide, DCVTNIAE (pep 331), with the same amino acid composition. NB100 cells were incubated separately with both peptides before being seeded on plates containing ADAM 23. As shown in Figure 9B, a significant loss of adherent cells was detected with samples incubated with pep 330 (~75%). In contrast, this effect could not be observed in samples incubated with the scrambled peptide derived from the same protein region (Figure 9B). These results indicate that human ADAM 23 specifically interacts with $\alpha v\beta 3$ through a protein region whose amino acid sequence is AVNECDIT and, therefore, in an RGD-independent manner.

DISCUSSION

Over the last few years the ADAM family of cellular disintegrins has grown considerably after the finding of a series of new members identified by using a variety of homology-cloning strategies. These cloning efforts have been largely stimulated by the putative dual functions of ADAMs as proteolytic enzymes and cell adhesion molecules. Recent studies have allowed characterization of the enzymatic properties and substrate specificity of several cellular disintegrins acting as proteinases, including TACE, MDC9, kuz/ADAM 10, ADAMTS-1, and ADAMTS-4 (Black *et al.*, 1997; Moss *et al.*, 1997; Kuno *et al.*, 1997; Pan and Rubin, 1997; Izumi *et al.*, 1998; Qi *et al.*, 1999; Roghani *et al.*, 1999; Tortorella *et al.*, 1999). However, their function as cell adhesion molecules is unclear in most cases. This is the case of ADAM 23 (MDC3), a recently described disintegrin whose expression in human tissues appears to be restricted to the brain (Sagane *et al.*, 1998). Structural analysis of the ADAM 23 amino acid sequence shows the presence of all protein domains characteristic of ADAMs, including metalloproteinase-like and disintegrin-like regions. However, the metalloproteinase-like domain of ADAM 23 lacks the three histidine residues as well as the glutamic acid residue forming part of the Zn-binding site characteristic of metalloproteinases (HEXXHXXGXXH) (Rawlings and Barrett, 1995). Therefore, it was tempting to speculate that ADAM 23 could be exclusively involved in cell adhesion processes rather than in protease-mediated events (Blobel, 1997; Wolfsberg and White, 1997). Consistent with this proposal, in this work we have provided evidence that the disintegrin-like domain of ADAM 23 strongly promotes neuroblastoma cell adhesion in a dose-dependent manner. Maximal adhesion was detected in the presence of divalent cations such as Mg^{2+} or Mn^{2+} at physiological concentrations, but not Ca^{2+}. This finding agrees well with results from a number of studies of regulation of integrin activity by divalent cations, demonstrating that the activity of several integrins is stimulated by Mg^{2+} or Mn^{2+} (Elices *et al.*, 1991; Luque *et al.*, 1994; Garratt and Humphries, 1995; Camper *et al.*, 1997). On the basis of these data, it was likely that the cell adhesion-promoting effects of ADAM 23 were mediated through interaction with some integrin receptor. In fact, by using a panel of CHO cells expressing different recombinant integrins, we have found that ADAM 23 specifically interacts with $\alpha v\beta 3$. In addition, the observed binding was inhibited by a function-blocking anti-$\alpha v\beta 3$ mAb but not by antibodies specific for other integrins. The specificity of the interaction between ADAM 23 and $\alpha v\beta 3$ was further confirmed by direct binding assays using purified proteins. Finally, we provide evidence that $\alpha v\beta 3$ promotes adhesion of HeLa cells transfected with an expression vector for full-length ADAM 23, thus extending the above observations performed with a recombinant protein exclusively containing the disintegrin-like domain of ADAM 23.

The finding that ADAM 23 is a ligand for $\alpha v\beta 3$ and promotes cell adhesion indicates that these properties are not exclusive of typical extracellular matrix adhesive proteins, being also shared by a variety of molecules with diverse biological functions, including thrombin (Bar-Shavit *et al.*, 1991), perlecan (Hayashi *et al.*, 1992), matrix metalloproteinase 2 (Brooks *et al.*, 1996), and basic fibroblast growth factor (Rusnati *et al.*, 1997). In this regard, it is also remark-

able that cells adherent to ADAM 23 exhibit differences in morphology when compared with those attached to characteristic extracellular matrix proteins such as fibronectin. These differences include the presence of numerous short protrusions resembling microspikes in cells grown on ADAM 23 as well as a distinct organization of actin filaments.

In this work, we have also tried to identify the molecular determinants mediating the observed interaction between ADAM 23 and αvβ3. At present, the best characterized disintegrin domains are those derived from snake venom proteins, which contain an RGD sequence at the tip of a flexible loop joining two strands of β-sheet protruding from the protein core (Adler *et al.*, 1993). This tripeptide interacts with platelet integrins inhibiting blood clot formation and favoring the generation of hemorrhages (Niewiarowski *et al.*, 1994). However, with the exception of metargidin (ADAM 15) (Krätzschmar *et al.*, 1996), all the other human ADAMs, including ADAM 23, lack this tripeptide motif in their disintegrin-like domains, which has led to the suggestion that they may promote rather than disrupt cell–cell interactions (Blobel, 1997; Wolfsberg and White, 1997). In fact, the disintegrin domain of different ADAMs has been proven to be essential in processes involving cell–cell interactions such as sperm–egg adhesion and muscle cell fusion (Blobel *et al.*, 1992; Almeida *et al.*, 1995; Yagami-Hiromasa *et al.*, 1995; Evans *et al.*, 1997; Yuan *et al.*, 1997; Inoue *et al.*, 1998). The results reported here for ADAM 23 and neuroblastoma cells are also consistent with this proposal. Thus, a peptide analogue of the ADAM 23 disintegrin loop specifically inhibited cell adhesion, whereas a scrambled ADAM 23 disintegrin loop peptide analogue did not. In addition, a mutant protein with a single alteration in a conserved residue present in the predicted binding loop of ADAM 23 had a significantly diminished ability to support neuroblastoma cell adhesion. Collectively, these results indicate that the receptor recognition and subsequent binding of ADAM 23 is mediated, at least in part, by the disintegrin loop present in this protein. In addition, the observation that the disintegrin-like domain of ADAM 23 produced in bacteria is enough to exert cell adhesive activity indicates that this protein does not require the remaining domains or the effective glycosylation of the disintegrin domain for performing this activity. Furthermore, the fact that ADAM 23 interaction with αvβ3 is independent of an RGD sequence distinguishes this cellular disintegrin from human metargidin (ADAM 15), which has also been reported to interact with αvβ3, albeit in an RGD-dependent manner (Zhang *et al.*, 1998; Nath *et al.*, 1999). It is also remarkable that the binding specificity of other disintegrins lacking RGD motifs is distinct from that determined herein for ADAM 23. Thus, the sperm surface protein ADAM 2 (fertilin β) interacts with the integrin α6β1 on mouse eggs and α6-transfected somatic cells (Almeida *et al.*, 1995; Yuan *et al.*, 1997, Chen *et al.*, 1999). Finally, the observation that αvβ3 integrins can bind to non-RGD sequences is not unprecedented, because such an interaction has been reported for other proteins, including matrix metalloproteinase 2, and basic fibroblast growth factor (Brooks *et al.*, 1996; Rusnati *et al.*, 1997), although in all cases the molecular basis of the observed interactions with αvβ3 remains unclear.

The interaction of ADAM 23 with αvβ3 may be related to the biological and/or pathological functions of this disintegrin. On the basis of data reported in this work demonstrating that ADAM 23 may promote adhesion of cells of neural origin, together with the predominant expression of ADAM 23 in the human brain in both fetal and adult stages, it is tempting to speculate that this protein could play some specialized role in the development and/or maintenance of neural functions. It is well known that development of the nervous system involves an orderly set of connections between the different parts of the system through the outgrowth of cellular protusions to create a functional network that is extremely complex. Axons and dendrites extend from the cell bodies by means of growth cones, which travel along precisely specified paths to connect with a concrete target cell with which they are going to synapse. Neurons of different functional classes show distinctive surface characteristics that determine specific contact interactions with other cell surfaces, especially from glial cells, and with components of the extracellular matrix. Such interactions are of major importance for leading neuronal growth cones toward their targets along precisely specified routes (Reichardt and Tomaselli, 1991; Tessier-Lavigne and Goodman, 1996; Shibata *et al.*, 1998; Davenport *et al.*, 1999). ADAM 23 could modulate some of these interactions through its ability to interact with αvβ3 in a manner similar to that found in the in vitro studies with neuroblastoma cells described in this work. Consistent with this, αvβ3 is abundantly expressed in the radial glial cells during mouse development and has been proposed to play an important role in the facilitation of neuronal migration within the central nervous system (Hirsch *et al.*, 1994).

In addition to potential roles of ADAM 23 in normal processes, the results here reported for ADAM 23 and neuroblastoma cells also suggest that this cellular disintegrin can favor tumor progression through the facilitation of integrin-mediated cell–cell interactions (Varner and Cheresh, 1996; Ruoslahti, 1997). Consistent with this, αvβ3 has been shown to be involved in the progression of melanoma and the induction of neovascularization by tumor cells (Seftor *et al.*, 1992; Brooks *et al.*, 1994). Similarly, the expression of integrin αvβ3 in undifferentiated neuroblastoma cells in vivo has been proposed to contribute to the rapid growth of these tumors and their tendency to metastasize (Gladson *et al.*, 1996). Finally, preliminary analysis of the nature of the signaling cascades initiated upon ADAM 23 binding to αvβ3 appear to indicate that this interaction results in the induction of active matrix metalloproteinases (S. Cal and C. López-Otín, unpublished results). As previously reported in other systems (Werb *et al.*, 1989; Riikonen *et al.*, 1995; Pilcher *et al.*, 1997; Lochter *et al.*, 1999) these proteolytic enzymes could act as effector molecules modifying the surrounding of the involved cells and facilitating further migration of tumor cells. The availability of recombinant ADAM 23 will open future studies directed to clarify its role in the context of other proteins involved in cell–cell or cell–matrix interaction processes taking place during the development and maintenance of neural functions, as well as in the alterations occurring in the metastatic events of tumor cells.

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