MDC9, a Widely Expressed Cellular Disintegrin Containing Cytoplasmic SH3 Ligand Domains

Gisela Weskamp, Jörn Krätzschmar, Martha S. Reid, and Carl P. Blobel

Cellular Biochemistry and Biophysics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021

Abstract. Cellular disintegrins are a family of proteins that are related to snake venom integrin ligands and metalloproteases. We have cloned and sequenced the mouse and human homologue of a widely expressed cellular disintegrin, which we have termed MDC9 (for metalloprotease/disintegrin/cysteine-rich protein 9). The deduced mouse and human protein sequences are 82% identical. MDC9 contains several distinct protein domains: a signal sequence is followed by a prodomain and a domain with sequence similarity to snake venom metalloproteases, a disintegrin domain, a cysteine-rich region, an EGF repeat, a membrane anchor, and a cytoplasmic tail. The cytoplasmic tail of MDC9 has two proline-rich sequences which can bind the SH3 domain of Src, and may therefore function as SH3 ligand domains. Western blot analysis shows that MDC9 is an ~84-kD glycoprotein in all mouse tissues examined, and in NIH 3T3 fibroblast and C2C12 myoblast mouse cell lines. MDC9 can be both cell surface biotinylated and ^{125}I-labeled in NIH 3T3 mouse fibroblasts, indicating that the protein is present on the plasma membrane. Expression of MDC9 in COS-7 cells yields an 84-kD protein, and immunofluorescence analysis of COS-7 cells expressing MDC9 shows a staining pattern that is consistent with a plasma membrane localization. The apparent molecular mass of 84 kD suggests that MDC9 contains a membrane-anchored metalloprotease and disintegrin domain. We propose that MDC9 might function as a membrane-anchored integrin ligand or metalloprotease, or that MDC9 may combine both activities in one protein.

Cellular disintegrins are a family of proteins that are related to snake venom disintegrins and metalloproteases. Disintegrins were first isolated from snake venom as short soluble peptides that bind with high affinity to the platelet integrin gpIIb/IIIa, thereby competitively inhibiting platelet aggregation (14, 17, 18, 23, 28, 37). The first recognized cellular disintegrin was the guinea pig sperm protein fertilin (previously referred to as PH-30), which mediates sperm-egg membrane binding and fusion (7, 8, 29, 35, 43). Additional cellular disintegrin sequences have been reported in the mouse (41), in mouse monocytes (49), as potential myoblast fusion protein (48), in rat and monkey epididymal epithelial cells (34), and in testis of different species (4, 16, 32–34, 46). Another cellular disintegrin was reported as a potential breast cancer tumor suppressor (11), and more partial and complete sequences have been deposited in the GenBank database (45). The family of known cellular disintegrins thus continues to expand.

Currently, the best characterized member of this protein family is the sperm-surface protein fertilin, which has been implicated in sperm-egg membrane binding and fusion. Fertilin is an integral membrane complex of two distinct cellular disintegrins, α and β (7, 8, 44), that are both made as larger precursors, and are processed at different stages of sperm maturation. The α subunit of fertilin is processed in the testis, and fertilin β is processed in the epididymis, where its cleavage correlates with the acquisition of fertilization competence in epidydimal sperm (7). Guinea pig sperm-egg membrane fusion can be inhibited by a monoclonal antibody against fertilin (35), as well as with peptides corresponding to the predicted fertilin integrin-binding domain (29), and mouse fertilin most likely binds to the α6β1 integrin on mouse eggs (3).

The known functions of fertilin and snake venom disintegrins raise intriguing ideas about the possible physiological role of the newly discovered cellular disintegrins. Snake venom disintegrins are high-affinity integrin ligands, and there is substantial evidence that fertilin is also an integrin ligand. Integrins (1, 19, 20, 36) are found on virtually every cell type, and mediate a variety of cell–cell and cell–matrix interactions. If other cellular disintegrins are

also integrin ligands, they may play an important role in some of these processes.

Here we report the cloning and cDNA sequencing, and the initial biochemical characterization of a widely expressed cellular disintegrin referred to as metalloprotease/disintegrin/cysteine-rich protein 9 (MDC9).[1] MDC9 is highly conserved between mouse and human, containing all protein domains that are found in other cellular disintegrins, and also includes a proline-rich cytoplasmic tail that may function as an Src homology 3 ligand domain. The apparent molecular mass of 84 kD in all tissues and cells examined suggests that mouse MDC9 contains a membrane-anchored metalloprotease and disintegrin domain. These results are discussed in the context of the predicted role of MDC9 in cell–cell interactions.

Materials and Methods

Animals and Reagents

Reagents, including *Taq* polymerase, were obtained from Boehringer Mannheim (Indianapolis, IN), unless indicated otherwise. Mice (nontransgenic animals) were obtained from the transgenic breeding facility of the Sloan Kettering Institute (New York, NY). Rabbits were purchased from Hazelton (Denver, PA). Radiolabeled nucleotides were supplied by Amersham Corp. (Arlington Heights, IL).

cDNA Cloning

A sequence tag encoding the partial disintegrin domain of MDC9 (sequence tag No. 16-2 (41)) was used to screen a mouse lung cDNA library (Stratagene, La Jolla, CA) under high stringency conditions as described (8), which yielded three identical clones with inserts of 2,330 bp. One cDNA clone was sequenced completely (Sequenase, USB, Cleveland, OH) in both orientations by a primer walk. Since the cDNA insert size of 2.33 kb did not match that observed for the mRNA (~4 kb), we constructed a new mouse lung cDNA library (Stratagene ZAP cDNA library synthesis kit, according to the manufacturer's instructions, see also reference 24). The unamplified cDNA library was screened under high stringency conditions with the partial 2.33 kb MDC9 clone, and yielded more than 100 positive plaques in the primary screen. PCR analysis of 80 positive plaques with an antisense primer that annealed close to the 5' end of the 2.33 kb clone, and the vector specific T3 primer, revealed that the cDNA of 10 clones extended between 800 and 850 bp past the 5' end of the shorter 2.33 kb clone. One cDNA clone was purified, subjected to in vitro excision, and sequenced as described above. This clone contained a cDNA insert of 3,851 bp, encoding for an open reading frame of 845 amino acid residues (GenBank accession No. U41765).

To clone the full-length cDNA for human MDC9, we synthesized a human cDNA library from MDA-MB-468 mammary epithelial carcinoma cells (ATCC HTB-132) as described above. The MDA-MB-468 cDNA library was screened with the full-length cDNA of mouse MDC9, and more than 50 positive plaques were identified. Clones with the longest 5' extension were identified by PCR, (see above), using as antisense primer the most 5' sequence of a partial human MDC9 sequence (GenBank accession No. D14665). One clone with a cDNA insert of 3,866 bp was sequenced on both strands, and contained an open reading frame of 819 amino acid residues (GenBank accession No. U41766). cDNA- and translated-protein sequences were analyzed using MacVector software (Kodak IBI, New Haven, CT). Sequence alignments and comparisons among cellular disintegrin proteins was performed using DNA STAR MEGALIGN software.

Northern Blot

Total RNA was isolated from mouse lung, heart, liver, lung, skeletal muscle, brain, spleen, kidney and testis, and from C2C12 mouse muscle cells

as described (9). For each tissue, 15 μg of RNA were run per lane, and the integrity of the ribosomal RNA of each sample was monitored on a separate gel. Human tissue Northern blots were obtained from Clontech (Palo Alto, CA). Northern blot analysis was performed with the appropriate ^{32}P-labeled mouse or human MDC9 cDNA probe under high stringency as previously described (41).

Blot Overlay with Biotinylated SH3 Domains of Src and Abl

Three separate gst-fusion proteins containing different portions of the mouse MDC9 cytoplasmic tail were constructed by PCR using gene-specific primers with added restriction sites to allow direct fusion with gst. Construct P1 encoded for amino acid residues 808 to 824 and included the potential SH3 ligand domain RPPPPQP, and construct P2 contained amino acid residues 825 to 845, including the second potential SH3 ligand domain RPAPAPP. In construct P1+2, both SH3 ligand domains were combined by a fusion with amino acid residues 808 to 845. The cDNA for each construct was sequenced to rule out any mutations possibly introduced by PCR. All three fusion proteins, and gst alone, were expressed in BL26 cells (Novagen, Madison, WI). To isolate the fusion proteins, bacteria were disrupted in a French press homogenizer, and gst-fusion proteins were allowed to bind to a glutathione matrix (Pharmacia LKB Biotechnology, Piscataway, NJ), and eluted with sample loading buffer. As probes for the SH3 ligand domain, gst-fusion proteins with either the SH3 domain of Src or Abl (kindly supplied by Dr. Margaret Chao), were isolated as described above, and biotinylated with NHS-LC-biotin (Pierce, Rockford, IL) following the manufacturer's instructions. A blot of the biotinylated Src- and Abl-SH3 fusion proteins was probed with HRP-labeled streptavidin (Pierce), and the level of biotinylation was found to be comparable between the two proteins (not shown). For the actual blot overlay experiment, MDC9 gst-fusion proteins were run on a 12% polyacrylamide gel and transferred to nitrocelluose. After blocking with 5% dry milk in water, the blot was incubated with the biotinylated Src- or Abl-SH3 fusion proteins (0.1 μg/ml), followed by incubation with HRP-labeled streptavidin. Bound HRP streptavidin was detected by chemiluminescence (ECL, Amersham) and exposure of Kodak XAR autoradiography film (New Haven, CT).

Antibodies

Polyclonal antisera were raised against a gst-fusion protein with the 114 COOH-terminal amino acid residues of the cytoplasmic tail of mouse MDC9 (constructed by PCR as described above for shorter gst-cytotail fusion proteins). Ribi adjuvant (RIBI Immunochemical Research Inc.) was used for immunizations according to established protocols (15). To demonstrate that the MDC9-cyto IgG specifically recognize MDC9, we attempted to affinity purify antibodies using the gst-fusion protein coupled to CNBr-activated Sepharose beads (Pharmacia Biotech). Although several different elution conditions were tested, we were unable to recover sufficient amounts of affinity purified IgG. As an alternative control for the specificity of the gst-cytotail antibodies, we immunodepleted protein A purified gst-cyto IgG of antibodies reacting with gst alone, or of antibodies reacting with the gst-cyto fusion protein, using gst or gst-cytotail fusion protein coupled to CNBr-activated CL4B beads. Immunodepletion of IgG reactive with gst or with the gst-cyto fusion protein was confirmed by probing Western blots of gst or gst-cyto fusion proteins.

Western Blot Analysis

Mouse tissues were dissected immediately after euthanasia (performed according to the guidelines of the American Veterinary Society), and homogenized in 10 ml lysis buffer per gram of tissue (1% NP-40, 1 mM EDTA, in PBS and protease inhibitors [7]) using a Polytron homogenizer (Kinematica, Littau, Switzerland). Mouse sperm were isolated from the distal cauda epididymis as described (35), washed in PBS, and lysed in lysis buffer at a concentration of 5×10^7 sperm per ml. NIH 3T3 mouse fibroblasts, and C2C12 mouse myoblasts were lysed at a concentration of 4×10^6 cells per ml. All lysates were spun at 27,000 g for 30 min, and the supernatant was incubated with 1 ml Con A–Sepharose (Pharmacia) per 10 ml extract for 1 h at 4°C. Bound glycoproteins from all tissues were eluted with 1 M α-methyl-D-mannose (Sigma Chem. Co., St. Louis, MO) in PBS, 1% NP-40, 5 mM EDTA, and protease inhibitors, for 10 min at 37°C. Sample buffer was added to the eluted glycoproteins, and all samples were heated to 95°C for 5 min, with or without 50 mM DTT, as indicated. Gly-

---

1. *Abbreviations used in this paper:* MDC9, metalloprotease/disintegrin/cysteine-rich protein 9; SH3, Src homology 3.

coproteins from mouse sperm, C2C12 mouse myoblasts, and NIH 3T3 fibroblast were eluted by boiling in sample loading buffer. Proteins were separated by SDS-PAGE (25), and transferred to nitrocellulose (Schleicher and Schuell, Keene, NH) using a semidry blotting apparatus (E and K, Saratoga, CA). Blotted protein samples were treated as described for Western blot analysis (7), except that a horseradish peroxidase–coupled secondary antibody (Promega Biotech, Madison, WI) was used in combination with a chemiluminescence detection system (ECL, Amersham) and Kodak XAR autoradiography film.

Expression of MDC9 in COS-7 Cells

The full-length gene for mouse MDC9 was cloned into a pcDNA3 expression vector (Invitrogen, San Diego, CA). MDC9 cDNA was transfected into COS-7 cells (kindly provided by Dr. M. Jasin) using lipofectin (GIBCO/BRL) to obtain a transiently transfected culture. Expression of MDC9 in COS-7 cells was monitored by Western blot as described above.

Cell Surface Labeling

NIH 3T3 mouse fibroblasts were biotinylated with the nonmembrane-permeable biotinylation reagent NHS-LC-biotin (Pierce) as described in reference 22, except that the cells were not lysed and removed from the dish until after the 45-min biotinylation reaction on ice, and the subsequent quenching of the labeling reaction with 0.1 M glycine. Cell surface iodination was performed by incubating a confluent 10-cm tissue culture dish of NIH 3T3 fibroblasts with 2 ml PBS containing 120 µg lactoperoxidase, 60 µl of 0.03% fresh H_2O_2, and 1 mCi ^{125}I at 4°C for 10 min. The reaction was terminated by addition of 10 ml PBS, 5 mM KI. Extracts of biotin-labeled or ^{125}I-labeled NIH 3T3 cells were immunoprecipitated with MDC9-specific antibodies, or control antibodies as described in reference 42. Immunoprecipitated ^{125}I-labeled material was run on SDS-PAGE, which was dried and used to expose Kodak XAR film by autoradiography, whereas immunoprecipitated biotinylated material was transferred to nitrocellulose, probed with horse radish peroxidase–labeled streptavidin, and detected using a chemiluminscent detection system as described above.

Immunofluorescence Staining

COS-7 cells transfected with MDC9 were grown on 8-well chamber slides (Nunc), fixed with 4% paraformaldehyde, and permeabilized with 1% NP-40 in PBS. MDC9-cyto antibodies, or gst-cyto–depleted control antibodies were added at a concentration of 1:150 in PBS, 0.2% NP-40, followed by a secondary rhodamine-labeled goat anti–rabbit antibody (Boehringer Mannheim Corp., Indianapolis, IN).

Results

Cloning and Sequencing of Mouse and Human MDC9 cDNA

In a previous study, the polymerase chain reaction was used to demonstrate that several characteristic sequence tags for cellular disintegrin proteins exist in the mouse (41). A cDNA clone of 3,851 bp coding for a full-length open reading frame (including sequence tag 16#2 [41]) was isolated from a mouse lung cDNA library as described in Materials and Methods. The protein encoded by this cDNA was termed MDC9, for metalloprotease disintegrin cysteine-rich protein 9. The deduced protein sequence of mouse MDC9 contains 845 amino acid residues (Fig. 1 A) including a predicted hydrophobic transmembrane domain (Fig. 1 B) and six potential N-linked glycosylation sites. The length of the MDC9 cDNA insert is consistent with the size of MDC9 mRNA, which is close to 4 kb as assayed by Northern blot analysis (Fig. 2). Mouse MDC9 is highly related to a partial human cDNA sequence deposited in GenBank (D14665; HUMORF_09). To compare mouse MDC9 with the full-length human MDC9, we cloned and sequenced human MDC9 from a cDNA library

constructed from MDA-MB-468 human mammary epithelial cancer cells. The longest human MDC9 clone had an insert of 3,866 bp with an open reading frame encoding for a protein with 819 amino acid residues.

An alignment of the deduced protein sequences of human and mouse MDC9 is shown in Fig. 1 A. The human and mouse MDC9 proteins are 82% identical, and contain all protein domains that are characteristic of cellular disintegrins. A signal sequence is followed by a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich region, an EGF repeat, a transmembrane domain, and a cytoplasmic tail. A comparison of mouse MDC9 with selected known cellular disintegrin proteins revealed an overall sequence similarity of 29% with guinea pig fertilin β, 27.5% with guinea pig fertilin α (8, 44), 20.5% with mouse MS2 (49), and 20.6% with rat EAPI (34) (data not shown, for examples of cellular disintegrin sequence alignments see references 8, 44, 45).
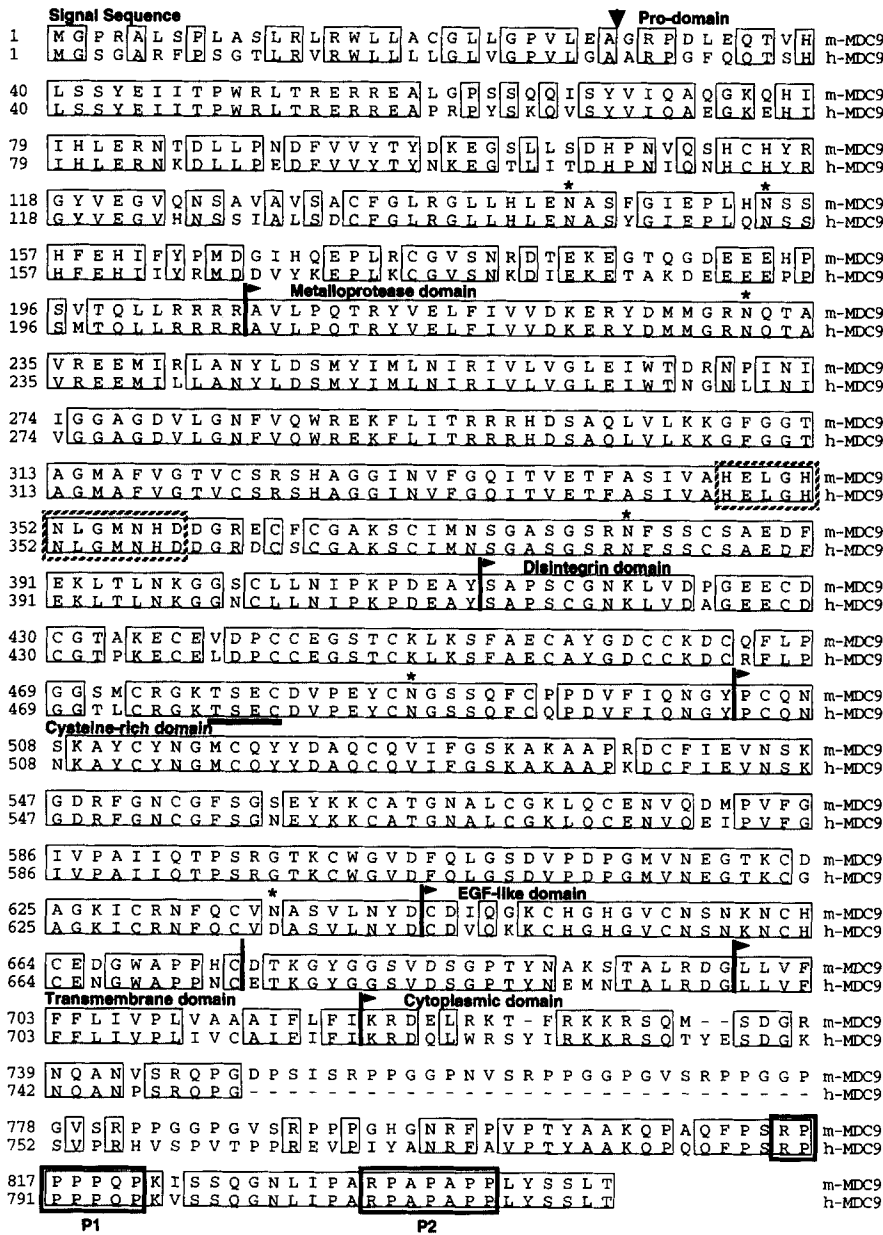
The hydrophobicity plot of mouse MDC9 indicates that, in addition to a hydrophobic transmembrane domain, a short hydrophobic region is present in precisely the same relative position as the predicted fusion peptide of fertilin α (Fig. 1 B). The short hydrophobic segment of both mouse MDC9 (not shown) and fertilin α can be modeled as amphipathic α helix (8). However, unlike guinea pig fertilin α, which is only expressed in testis, both human and mouse MDC9 are expressed in all tissues examined by Northern blot analysis (Fig. 2).

Western Blot and Immunofluorescence Analysis

To analyze the domain organization of MDC9 in mouse tissues and cell lines, we raised antibodies against a gst-fusion protein with the cytoplasmic tail of MDC9. For all experiments involving MDC9-reactive antibodies, we used antibodies raised against the gst-MDC9-cytoplasmic tail, and depleted of IgG that bind to the gst moiety only (referred to as MDC9-cyto IgG, see Materials and Methods). As control IgG, we depleted an identical MDC9 gst-cyto-tail IgG sample of all antibodies reacting with the gst-MDC9-cyto fusion protein.

Since mouse MDC9 was cloned from a lung cDNA library, the initial characterization of MDC9 was performed on mouse lung tissue. A Western blot of a mouse lung extract probed with MDC9-cyto IgG (Fig. 3 A, lane 1) showed a strong band of 84 kD, and two weaker bands of 115 and 60 kD. As mouse MDC9 is predicted to have six N-linked glycosylation sites, it was expected to bind to the lectin Con A. In a mouse lung extract depleted of glycoproteins with Con A, the bands of 84 and 115 kD were not visible, whereas the 60-kD band was still detectable (Fig. 3 A, lane 2). Both the 84-kD and 115-kD bands were recovered from Con A by competitive elution with α-methyl-D-mannose (Fig. 3 A, lane 3). Due to the high cysteine content of MDC9 (45 extracellular cysteine residues), reduced and nonreduced MDC9 should show a clearly different mobility on SDS-PAGE. In a sample of mouse lung glycoproteins analyzed under nonreducing conditions, the MDC9-cyto IgG recognized a band of 76 kD, and a weaker 115-kD band (Fig. 3 A, lane 5). Fig. 3 A, lane 4 contains nonreduced mouse lung glycoproteins run adjacent to the reduced sample in lane 3. Therefore, proteins in the left part

A

Signal Sequence   ▼ Pro-domain

```
1    M G P R A L S P L A S L R L R W L L A C G L L G P V L E A G R P D L E Q T V H   m-MDC9
1    M G S G A R F P S G T L R V R W L L L L G L V G P V L G A A R P G F Q Q T S H   h-MDC9

40   L S S Y E I I T P W R L T R E R R E A L G P S S Q Q I S Y V I Q A Q G K Q H I   m-MDC9
40   L S S Y E I I T P W R L T R E R R E A P R P Y S K Q V S Y V I Q A E G K E H I   h-MDC9

79   I H L E R N T D L L P N D F V V Y T Y D K E G S L L S D H P N V Q S H C H Y R   m-MDC9
79   I H L E R N K D L L P E D F V V Y T Y N K E G T L I T D H P N I Q N H C H Y R   h-MDC9

118  G Y V E G V Q N S A V A V S A C F G L R G L L H L E N A S F G I E P L H N S S   m-MDC9
118  G Y V E G V H N S S I A L S D C F G L R G L L H L E N A S Y G I E P L Q N S S   h-MDC9

157  H F E H I F Y P M D G I H Q E P L R C G V S N R D T E K E G T Q G D E E E H P   m-MDC9
157  H F E H I I Y R M D D V Y K E P L K C G V S N K D I E K E T A K D E E E E P P   h-MDC9

                            ► Metalloprotease domain
196  S V T Q L L R R R R A V L P Q T R Y V E L F I V V D K E R Y D M M G R N Q T A   m-MDC9
196  S M T Q L L R R R R A V L P Q T R Y V E L F I V V D K E R Y D M M G R N Q T A   h-MDC9

235  V R E E M I R L A N Y L D S M Y I M L N I R I V L V G L E I W T D R N P I N I   m-MDC9
235  V R E E M I L L A N Y L D S M Y I M L N I R I V L V G L E I W T N G N L I N I   h-MDC9

274  I G G A G D V L G N F V Q W R E K F L I T R R R H D S A Q L V L K K G F G G T   m-MDC9
274  V G G A G D V L G N F V Q W R E K F L I T R R R H D S A Q L V L K K G F G G T   h-MDC9

313  A G M A F V G T V C S R S H A G G I N V F G Q I T V E T F A S I V A H E L G H   m-MDC9
313  A G M A F V G T V C S R S H A G G I N V F G Q I T V E T F A S I V A H E L G H   h-MDC9

352  N L G M N H D D G R E C F C G A K S C I M N S G A S G S R N F S S C S A E D F  m-MDC9
352  N L G M N H D D G R D C S C G A K S C I M N S G A S G S R N F S S C S A E D F  h-MDC9

                         ► Disintegrin domain
391  E K L T L N K G G S C L L N I P K P D E A Y S A P S C G N K L V D P G E E C D   m-MDC9
391  E K L T L N K G G N C L L N I P K P D E A Y S A P S C G N K L V D A G E E C D   h-MDC9

430  C G T A K E C E V D P C C E G S T C K L K S F A E C A Y G D C C K D C Q F L P   m-MDC9
430  C G T P K E C E L D P C C E G S T C K L K S F A E C A Y G D C C K D C R F L P   h-MDC9

469  G G S M C R G K T S E C D V P E Y C N G S S Q F C P P D V F I Q N G Y P C Q N   m-MDC9
469  G G T L C R G K T S E C D V P E Y C N G S S Q F C Q P D V F I Q N G Y P C Q N   h-MDC9

     Cysteine-rich domain
508  S K A Y C Y N G M C Q Y Y D A Q C Q V I F G S K A K A A P R D C F I E V N S K   m-MDC9
508  N K A Y C Y N G M C Q Y Y D A Q C Q V I F G S K A K A A P K D C F I E V N S K   h-MDC9

547  G D R F G N C G F S G S E Y K K C A T G N A L C G K L Q C E N V Q D M P V F G   m-MDC9
547  G D R F G N C G F S G N E Y K K C A T G N A L C G K L Q C E N V Q E I P V F G   h-MDC9

586  I V P A I I Q T P S R G T K C W G V D F Q L G S D V P D P G M V N E G T K C D   m-MDC9
586  I V P A I I Q T P S R G T K C W G V D F Q L G S D V P D P G M V N E G T K C G   h-MDC9

                         ► EGF-like domain
625  A G K I C R N F Q C V N A S V L N Y D C D I Q G K C H G H G V C N S N K N C H   m-MDC9
625  A G K I C R N F Q C V D A S V L N Y D C D V Q K K C H G H G V C N S N K N C H   h-MDC9

664  C E D G W A P P H C D T K G Y G G S V D S G P T Y N A K S T A L R D G L L V F   m-MDC9
664  C E N G W A P P N C E T K G Y G G S V D S G P T Y N E M N T A L R D G L L V F   h-MDC9

     Transmembrane domain          ► Cytoplasmic domain
703  F F L I V P L V A A A I F L F I K R D E L R K T - F R K K R S Q M - - S D G R   m-MDC9
703  F F L I V P L I V C A I F I F I K R D Q L W R S Y I R K K R S Q T Y E S D G K   h-MDC9

739  N Q A N V S R Q P G D P S I S R P P G G P N V S R P P G G P G V S R P P G G P   m-MDC9
742  N Q A N P S R Q P G - - - - - - - - - - - - - - - - - - - - - - - - - - - -   h-MDC9

778  G V S R P P G G P G V S R P P P G H G N R F P V P T Y A A K Q P A Q F P S R P   m-MDC9
752  S V P R H V S P V T P P R E V P I Y A N R F A V P T Y A A K Q P Q Q F P S R P   h-MDC9

817  P P P Q P K I S S Q G N L I P A R P A P A P P L Y S S L T                       m-MDC9
791  P P P Q P K V S S Q G N L I P A R P A P A P P L Y S S L T                       h-MDC9
     P1                          P2
```
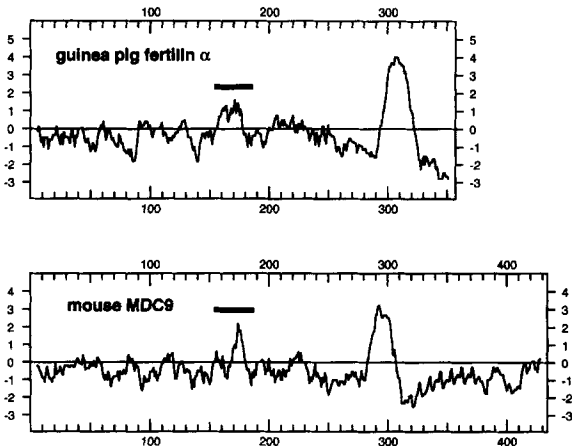
B

Figure 1. Comparison of the amino acid sequence of mouse and human MDC9. (A) Alignment of the mouse and human MDC9 amino acid sequences (produced with DNA STAR MEGA-LIGN). A potential signal peptidase cleavage site (39) is marked with a large arrowhead. The predicted NH_2 termini (44) of individual disintegrin protein domains are indicated by a vertical bar and a small horizontal arrowhead above the aligned sequences. The metalloprotease consensus sequence is indicated by a box with shaded outlines. The position of the predicted integrin-binding sequence TSEC within the disintegrin domain is underlined. Potential N-linked glycosylation sites of mouse MDC9 are marked with an asterisk. Cytoplasmic proline rich sequences that are possibly SH3 ligand domains are boxed and marked P1 and P2. (B) Hydrophobicity plots of mouse MDC9 and of guinea pig fertilin α beginning at the NH_2 terminus of the disintegrin domain (produced with DNA strider 1.1 software). A bar marks the position of the putative fusion peptide of fertilin α, and of the hydrophobic sequence in MDC9.
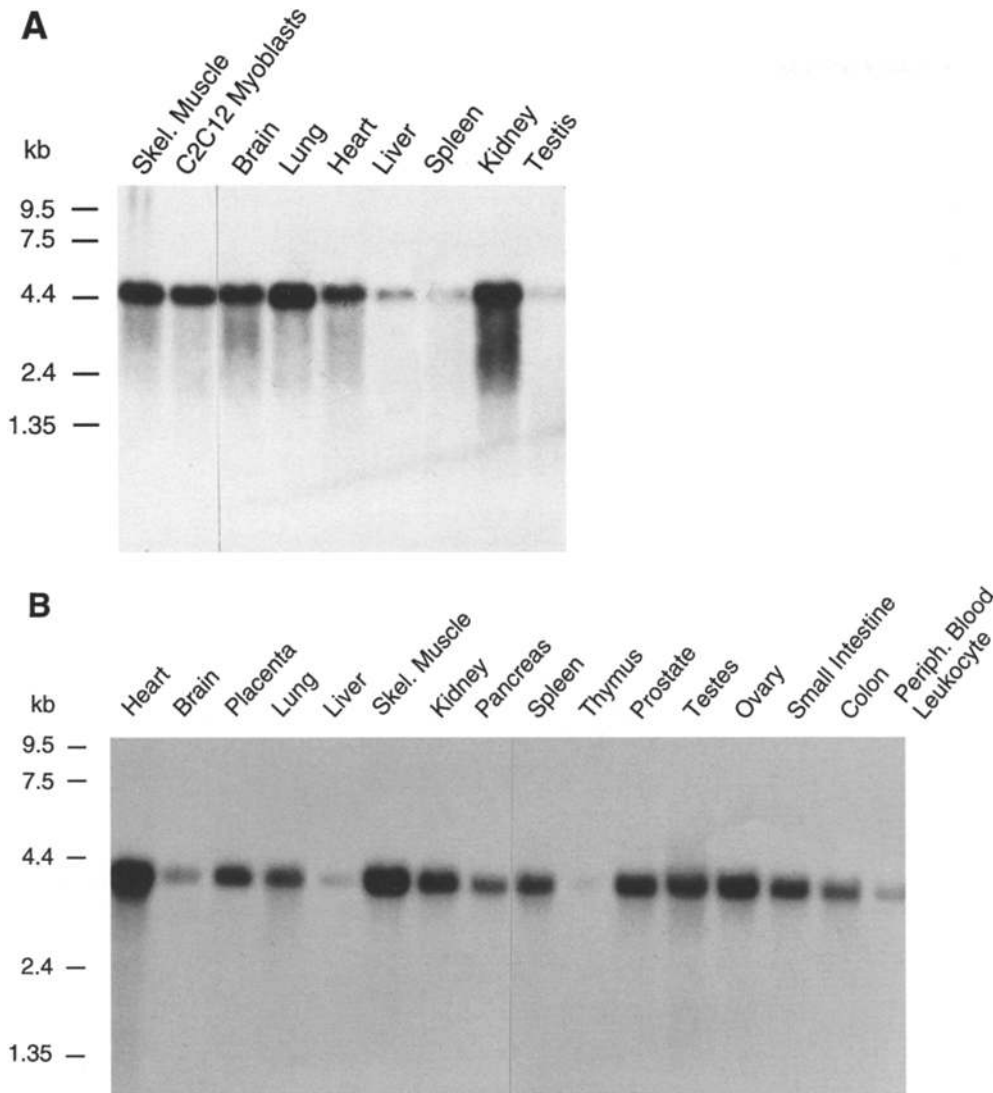
A

Skel. Muscle
C2C12 Myoblasts
Brain
Lung
Heart
Liver
Spleen
Kidney
Testis

kb

9.5 —
7.5 —

4.4 —

2.4 —

1.35 —

B

Heart
Brain
Placenta
Lung
Liver
Skel. Muscle
Kidney
Pancreas
Spleen
Thymus
Prostate
Testes
Ovary
Small Intestine
Colon
Periph. Blood Leukocyte

kb

9.5 —
7.5 —

4.4 —

2.4 —

1.35 —

Figure 2. Northern blot analysis of mouse and human MDC9 expression. Northern blots of mRNA isolated from (*A*) mouse tissues and proliferating C2C12 mouse myoblasts (15 μg total RNA loaded per lane) and (*B*) human tissues (Clontech human tissue Northern blots 1 and 2) were probed under high stringency conditions with the appropriate ^{32}P-labeled mouse or human cDNA. The mRNA tissue source is indicated above each lane.

of lane *4* are partially reduced, which allows the 76-kD band (Fig. 3 *A*, lane *5*, nonreduced sample) to be traced into the 84-kD band (Fig. 3 *A*, lane *3*, reduced sample). An identical blot probed with control IgG revealed no binding to the 76-kD (nonreduced) or 84-kD (reduced) band, whereas the 115-kD band was still visible (Fig. 3 *A, bottom panel*).

To analyze the expression and processing of MDC9 in different mouse tissues and cell lines, a Western blot of several Con A–purified mouse tissue extracts run under reducing conditions was probed with MDC9-cyto IgG, or control IgG. In all tissues examined, as well as in mouse sperm, NIH 3T3 fibroblasts and C2 muscle cells, an ∼84-kD protein was observed (Fig. 3 *B, top panel*). The relative tissue specific expression levels of the MDC9 protein (Fig. 3 *B, top panel*) and mRNA (Fig. 2 *A*) are different, which could result from a different detergent extractability of MDC9 depending on the tissue source, or could indicate that MDC9 expression is also regulated at the posttranscriptional level. The slight variations in apparent molecular weight might be explained by different carbohydrate modifications in different tissues. Both the NIH 3T3 and

C2C12 (47) mouse cell lines also contain a 120-kD band that is recognized by MDC9-cyto IgG, but not by control IgG, and may represent a small amount of MDC9 precursor protein.

As a further confirmation that the 84-kD protein recognized by MDC9-cyto IgG in mouse tissues and cells is indeed MDC9, we transfected MDC9 cDNA into COS-7 cells, and compared extracts of MDC9 expressing COS-7 cells with mouse lung glycoproteins by Western blot (Fig. 3 *C, top panel*). Whereas extracts of nontransfected (Fig. 3 *C*, lane *4*), or vector-transfected COS-7 cells (Fig. 3 *C*, lane *3*) did not contain an 84-kD band recognized by MDC9-cyto IgG, extracts of MDC9 expressing COS-7 cells (Fig. 3 *C*, lane *2*) contained a protein very similar to the 84-kD glycoprotein in mouse lung (Fig. 3 *C*, lane *1*). A weaker band of 65 kD, which is only visible in MDC9-transfected COS-7 cells, may represent a proteolytically processed form of MDC9. In an identical blot probed with control IgG the 84-kD and 65-kD bands were not visible (Fig. 3 *C, bottom panel*). The control IgG further demonstrate that the 60-kD band in lane *1*, and the 30-kD bands in lanes *2–4* are not specifically recognized by MDC9-cyto IgG. Based on
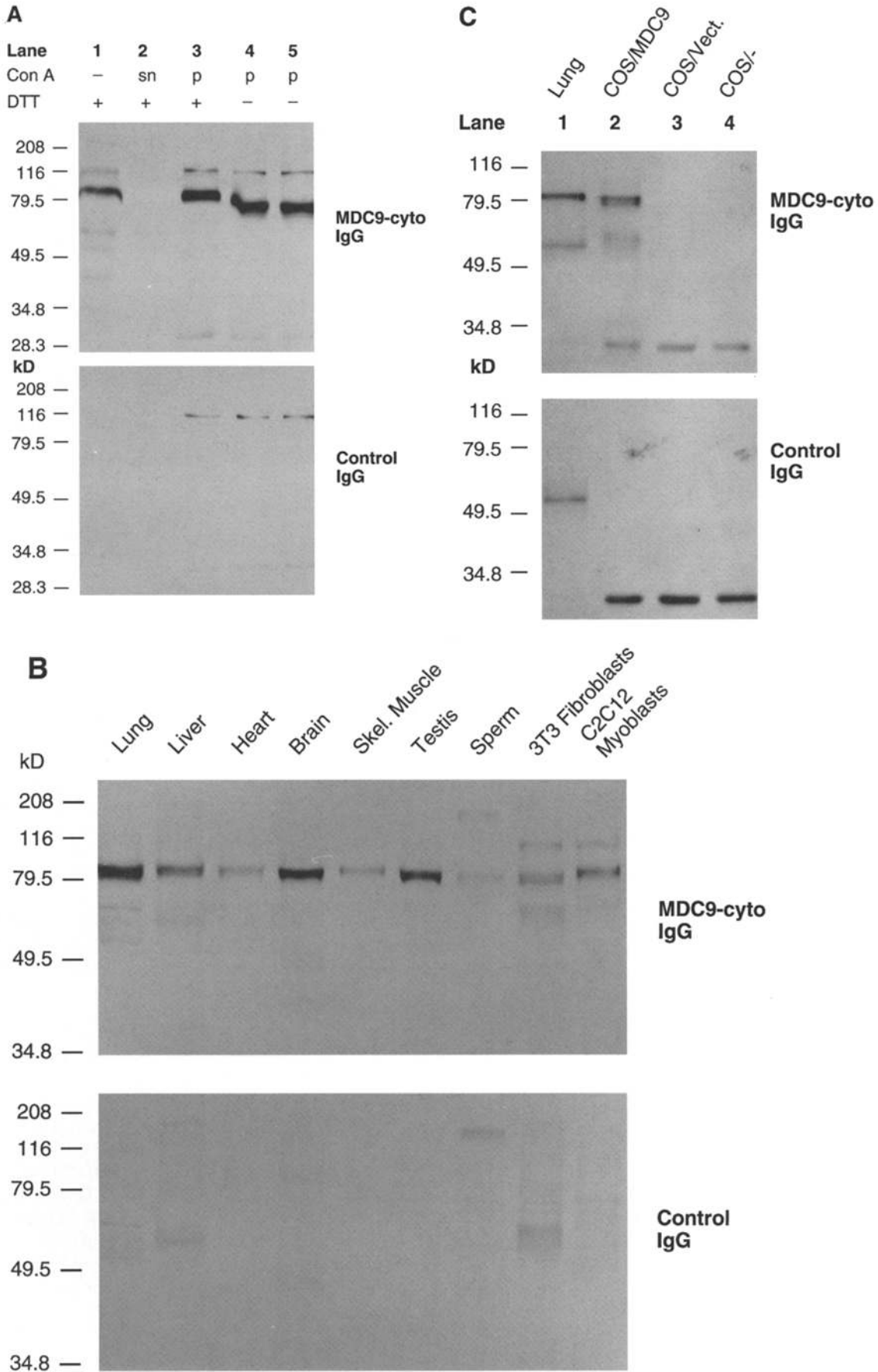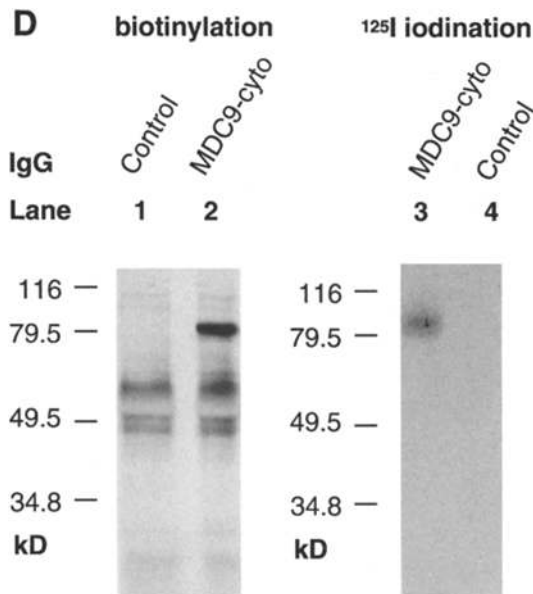
A

Lane	1	2	3	4	5
Con A	–	sn	p	p	p
DTT	+	+	+	–	–

208 —
116 —
79.5 —
49.5 —
34.8 —
28.3 —
kD

MDC9-cyto
IgG

208 —
116 —
79.5 —
49.5 —
34.8 —
28.3 —

Control
IgG

C

Lane	Lung	COS/MDC9	COS/Vect.	COS/-
	1	2	3	4

116 —
79.5 —
49.5 —
34.8 —
kD

MDC9-cyto
IgG

116 —
79.5 —
49.5 —
34.8 —

Control
IgG

B

Lung Liver Heart Brain Skel. Muscle Testis Sperm 3T3 Fibroblasts C2C12 Myoblasts

kD
208 —
116 —
79.5 —
49.5 —
34.8 —

MDC9-cyto
IgG

208 —
116 —
79.5 —
49.5 —
34.8 —

Control
IgG

Figure 3.

D biotinylation ^{125}I iodination

IgG

Lane   1   2   3   4

116 —   116 —

79.5 —   79.5 —

49.5 —   49.5 —

34.8 —   34.8 —

kD   kD

Figure 3. Western blot analysis and cell surface labeling of MDC9. Western blots of (*A*) mouse lung extracts, (*B*) mouse tissue extracts, and (*C*) extracts of COS-7 cells expressing MDC9, were probed with MDC9-cyto IgG prepared as described in Materials and Methods (*upper panel* in *A–C*), or with control IgG (*lower panel* in *A–C*). Mouse lung samples in *A* were loaded as follows: whole extract (lane *1*), extract supernatant (*sn*) after removal of glycoproteins with Con A (lane *2*), glycoproteins eluted from the Con A pellet (*p*) with 1 M α methyl-D-mannose as described in Materials and Methods (lanes *3–5*). Samples in lanes *1–3* were reduced before electrophoresis, whereas samples in lanes *4* and *5* were not. In *B* the source of Con A purified glycoproteins (mouse lung, liver, heart, brain, testis, muscle, sperm, C2C12 myoblasts, and NIH 3T3 fibroblasts) is indicated above each lane. 10 µl of Con A purified glycoproteins from each tissue (prepared as described in Materials and Methods) or mouse sperm glycoproteins from 10^7 sperm, from 5×10^5 C2C12 myoblasts, or 5×10^5 NIH 3T3 fibroblasts were loaded per lane. *C* shows a Western blot of mouse lung (lane *1*), MDC9 expressing COS-7 cells (lane *2*), vector-transfected COS-7 cells (lane *3*), and untransfected COS-7 cells (lane *4*). In *D*, extracts of surface biotinylated (lanes *1* and *2*), or ^{125}I-labeled (lanes *3* and *4*) NIH 3T3 fibroblasts were immunoprecipitated with MDC9-cyto IgG (lanes *2* and *3*), or with control IgG (lanes *1* and *4*). Biotinylated proteins were detected with streptavidin HRP as described in Materials and Methods, and ^{125}I-labeled proteins were detected by autoradiography.

the data presented above, we conclude that the 84-kD protein recognized on Western blots of mouse tissues under reducing conditions is MDC9.

Considering that cellular disintegrins are predicted to play a role in cell–cell interactions, MDC9 should be detectable at the cell surface. To confirm that MDC9 is present at the cell surface, NIH 3T3 fibroblasts were cell surface labeled by two different methods, biotinylation with the water soluble reagent NHS-LC-biotin, or ^{125}I iodination catalyzed by lactoperoxidase. Labeled NIH 3T3 cell extracts were immunoprecipitated with MDC9-cyto IgG, or control IgG. Fig. 3 *D* shows that a biotinylated protein (lane *2*), or a ^{125}I-labeled protein (lane *3*) of 84 kD can be immunoprecipitated from NIH 3T3 cells with the MDC9-

cyto IgG, but not with control IgG (Fig. 3 *D*, lanes *1* and *4*). Although both labeling methods confirm that MDC9 is indeed expressed on the cell surface of NIH 3T3 fibroblasts, immunofluorescence staining of fixed and permeabilized NIH 3T3 fibroblasts and C2C12 cells with the MDC9-cyto IgG did not reveal a clear cell surface staining. This result may be due to an insufficient amount of MDC9 on the plasma membrane for detection, or to obstruction of cytoplasmic epitopes. When immunofluorescence analysis with MDC9-cyto IgG was performed on COS-7 cells that were transiently transfected with MDC9, a clearly visible staining of transfected cells was observed. As shown in Fig. 4 *A*, staining extended into the cell boundary and into processes emanating from the labeled cell, consistent with the staining pattern of a plasma membrane protein. Nontransfected cells showed very little staining with MDC9-cyto IgG (Fig. 4 *C*), and no staining of either transfected (Fig. 4 *B*) or nontransfected cells (not shown) incubated with control IgG was apparent. These results suggest that, by independent assays, MDC9 is expressed at the cell surface of NIH 3T3 fibroblasts and of COS-7 cells.

Potential Src Homology 3 Ligand Domains

The sequence of the cytoplasmic tail of human and mouse MDC9 is relatively rich in proline residues, including two short sequences that closely resemble the consensus for Src homology 3 (SH3) ligand domains (Fig. 1 *A*) (2, 12, 26, 27, 38). To provide preliminary evidence that these proline-rich sequences might function as SH3 ligands, we blotted expressed gst-fusion proteins containing different portions of the MDC9 cytoplasmic tail onto nitrocellulose. Gst-chimeras with the SH3 domains of Src or Abl were used to overlay blotted MDC9 gst-fusion proteins. Fig. 5, bottom panel, shows a Ponceau S stained blot of expressed MDC9 gst-fusion proteins. Fig. 5, lanes *1* and *5* contain a gst-fusion protein with both potential SH3 ligands of the MDC9 cytoplasmic tail, lanes *2* and *6* contain a gst-fusion with only the first, and lanes *3* and *7* with only the second proline-rich sequence, whereas lanes *4* and *8* contain the gst protein alone. In Fig. 5, upper panel, lanes *1–4* of the same blot are overlayed with a biotinylated Src SH3-gst-fusion protein, which bound best to the construct containing both potential SH3 ligand domains. Comparably weaker binding of Src SH3-gst to either construct with just one proline-rich sequence was evident, while Src SH3-gst did not bind to gst alone. The Abl SH3 domain, which is known to bind different ligands than the Src SH3 domain (2, 12, 26) (see below), did not bind to the MDC9 cytotail-gst-fusion proteins (Fig. 5, lanes *5–8, upper panel*), consistent with a distinct binding specificity of the MDC9 SH3 ligand domains for different SH3 domains.

Discussion

The present study describes the cloning and initial biochemical characterization of the cellular disintegrin MDC9 (metalloprotease/disintegrin/cysteine-rich protein 9). Cellular disintegrins are a recently recognized protein family with at least 17 members in the mouse (16, 41, 45, 48, 49) (Weskamp, G., and C. Blobel, unpublished sequence tags), and a growing number of members in other species (4, 11,
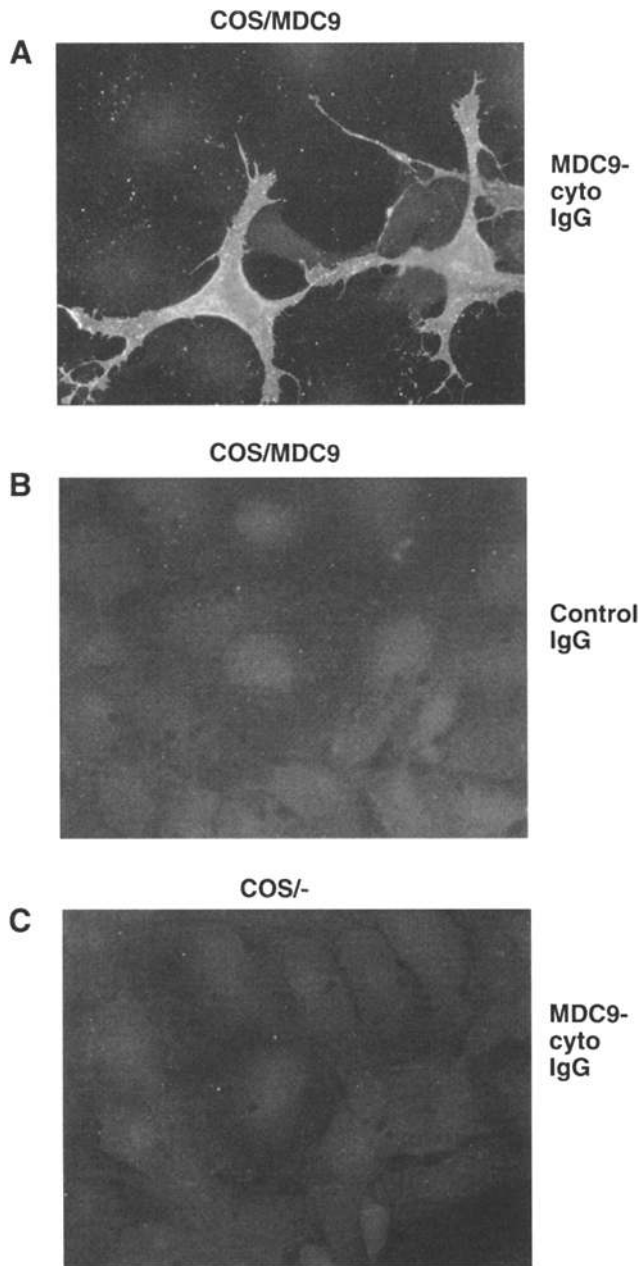
COS/MDC9 — A — MDC9-cyto IgG

COS/MDC9 — B — Control IgG

COS/- — C — MDC9-cyto IgG

Figure 4. Immunofluorescence staining of MDC9 expressing COS-7 cells. MDC9 expressing COS-7 cells were fixed with 4% paraformaldehyde, permeabilized with 1% NP-40, and incubated with MDC9-cyto IgG (*A*), or with control IgG (*B*), at a 1:150 dilution. Fixed and permeabilized vector-transfected (*control*) COS-7 cells incubated with MDC9 cytotail antibodies are shown in *C*. Bound antibodies were visualized with a Zeiss Axiophot microscope after addition of rhodamine-coupled secondary antibodies.

21, 32–34, 46). Since the currently best characterized cellular disintegrin, the guinea pig sperm protein fertilin, plays a critical role in sperm-egg membrane binding and fusion (29, 35), it has been proposed that other cellular disintegrins also mediate cell–cell or cell–matrix interactions (6, 41, 46).

Both human and mouse MDC9 contain all protein domains that are characteristic for cellular disintegrins (see above, and Fig. 1 *A*). Within the metalloprotease domain of MDC9, the zinc-binding consensus sequence HEXXH
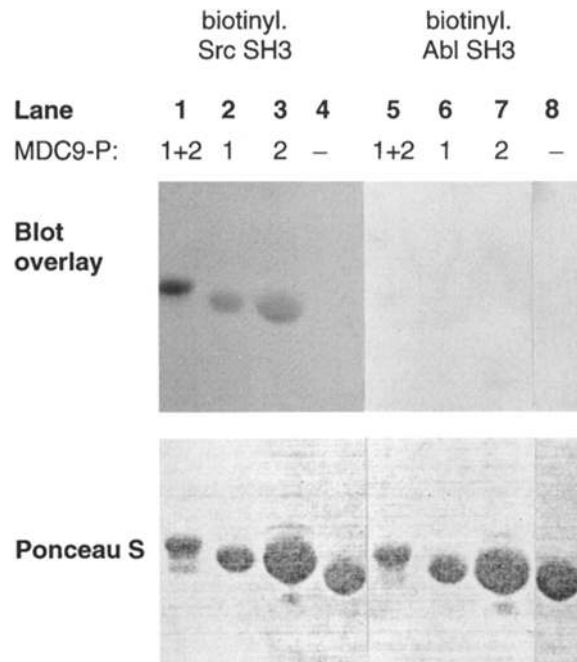
Figure 5. Binding of Src and Abl SH3 domains to MDC9 cytoplasmic tail sequences. Gst-fusion proteins with different parts of the mouse MDC9 cytoplasmic tail were blotted onto nitrocellulose and stained with *Ponceau S* (*lower panel*). Blotted proteins were probed with biotinylated gst-fusion proteins with the SH3 domains of Src (*upper panel*, lanes *1–4*) or Abl (*upper panel*, lanes *5–8*). In both panels, lanes *1* and *5* contain a gst-fusion protein with both proline-rich MDC9 cytoplasmic sequences (P1 and P2, bold type in Fig. 1 *A*), lanes *2* and *6* contain a fusion-protein with only P1, and lanes *3* and *7* with only P2. Lanes *4* and *8* contain gst as a control.

is in a conserved position compared to related snake venom metalloproteases (6, 44). Therefore, we predict that MDC9 can function as a metalloprotease, which may be regulated through a cysteine switch mechanism (5, 6, 44). The predicted cleavage site between the MDC9 pro- and metalloprotease domain contains four consecutive arginine residues, and thus a substrate sequence for serine proteases such as the pro-protein convertase furin (10, 13, 40). Furin is known to cleave and activate another prometalloprotease, stromelysin, between the prodomain and the catalytically active domain (31).

The disintegrin domain of MDC9 is highly related to RGD-type snake venom disintegrins (6, 8, 14). An important distinction is that, like all other presently reported cellular disintegrins, MDC9 does not contain an RGD sequence in the same relative position as the snake venom disintegrin RGD. The sequence found instead of the RGD in both mouse and human MDC9 is TSE, which is directly followed by an additional cysteine residue not present in RGD-type snake venom disintegrins, but is found in all other cellular disintegrins. Several integrins are known to bind to sequences other than RGD (20), and, although the effect of the additional cysteine residue on the folding and function of cellular disintegrins remains to be established, the sequence replacing the RGD in cellular disintegrins is predicted to function as an integrin ligand (6, 8). This hypothesis is supported by fertilization studies, where pep-

724

tides corresponding to the predicted integrin-binding sequence of fertilin β were shown to block sperm-egg fusion, whereas scrambled control peptides did not (3, 29). Furthermore, mouse fertilization can be blocked by antibodies against the integrin α6β1 (3).

The cysteine-rich region of MDC9 that follows the disintegrin domain includes a hydrophobic sequence in the same position, relative to conserved cysteine residues, as the predicted fusion peptide of guinea pig fertilin α (8).

Like the fusion peptide of fertilin α, the hydrophobic region of MDC9 can be modeled as an amphipathic helix, which raises the question whether MDC9 could also be a membrane fusion protein. However, since expression of MDC9 is not restricted to tissues involved in cell–cell fusion, but instead is ubiquitous, any potential function of MDC9 in cell–cell fusion would have to be strictly regulated. Alternatively, this hydrophobic region may serve a different function in MDC9, such as a role in protein folding or protein–protein interactions.

The most notable feature of the cytoplasmic sequences of mouse and human MDC9 are two conserved potential SH3 ligand domains (RPPPPQP, and RPAPAPP), which are similar to the Src SH3-binding consensus sequence RPLPXXP (2, 12, 26). Consistent with the predicted specificity of the MDC9 SH3 ligand domains, a biotinylated Src SH3 domain interacts with the MDC9 proline-rich sequences, whereas the Abl SH3 domain does not (2). The binding of Src SH3 to MDC9 proline-rich sequences suggests that these may indeed function as SH3 ligand domains, although it does not imply that Src is the actual ligand in vivo, since a leucine in position 3 is found in known Src-SH3 ligand domains (2, 38). SH3 domains are present in cytoskeletal proteins and in signaling molecules (30), and it is tempting to speculate that MDC9 may interact with cytoplasmic proteins. At least two other cellular disintegrins, mouse MS2 (49) and meltrin α (48), also contain similar cytoplasmic proline-rich sequences.

In characterizing MDC9, it was important to determine if its metalloprotease and/or disintegrin domains are membrane anchored. The functional implications are that a plasma membrane–anchored disintegrin domain could have a role in cell–cell interactions, whereas a proteolytically released disintegrin domain might function as a matrix-associated integrin ligand, or as a soluble integrin ligand which could possibly disrupt cell–cell or cell–matrix adhesion (41). Likewise, a membrane-anchored metalloprotease domain might have other roles and substrates than a soluble metalloprotease. In all mouse tissue or cell extracts that were analyzed by Western blot with MDC9-cyto IgG, a glycoprotein of close to 84 kD was revealed, indicating that MDC9 is not subject to different proteolytic processing events in these tissues. Because the predicted molecular weight of mouse MDC9 (after removal of the signal sequence) is 89, the apparent molecular weight of 84 suggests that, in most tissues and cells, MDC9 contains a membrane-anchored metalloprotease and disintegrin domain. MDC9 could be cell surface biotinylated and ^{125}I-iodinated on NIH 3T3 mouse fibroblasts, indicating that MDC9 is present on the cell surface of these cells. Furthermore, the immunofluorescence staining pattern of MDC9 expressing COS-7 cells was consistent with that of a cell surface protein.

In summary, we have isolated and sequenced cDNA clones encoding mouse and human MDC9, a widely expressed cellular disintegrin. The cytoplasmic domain of MDC9 contains potential SH3 ligand domains and may play a role in interactions with the cytoskeleton or with intracellular signaling molecules. The apparent molecular weight of MDC9 in different mouse tissues and cell lines suggests that it contains both membrane-anchored disintegrin and metalloprotease domains, and might therefore have a dual function as a metalloprotease and/or integrin ligand. We anticipate that further study of this widely expressed cellular disintegrin protein will lead to a better understanding of the role of members of this newly recognized protein family in cell–cell interactions.

References

1. Albelda, S.M., and C.A. Buck. 1990. Integrins and other cell adhesion molecules. FASEB. (Fed. Am. Soc. Exp. Biochem.) 4:2868–2880.
2. Alexandropoulos, K., G. Cheng, and D. Baltimore. 1995. Proline-rich sequences that bind to Src homology 3 domains with individual specificities. Proc. Natl. Acad. Sci. USA. 92:3110–3114.
3. Almeida, E.A. C., A.-P.J. Huovila, A.E. Sutherland, L.E. Stephens, P.G. Calarco, L.M. Shaw, A.M. Mercurio, A. Sonnenberg, P. Primakoff, D.G. Myles, et al. 1995. Mouse egg integrin α6β1 functions as a sperm receptor. Cell. 81:1095–1104.
4. Barker, H.L., A.C.F. Perry, R. Jones, and L. Hall. 1994. Sequence and expression of a monkey testicular transcript encoding tMDC I, a novel member of the metalloprotease-like, disintegrin-like, cysteine-rich (MDC) protein family. Biochem. Biophys. Acta. 1218:429–431.
5. Birkedal-Hansen, H. 1995. Proteolytic remodeling of extracellular matrix. Curr. Opin. Cell Biol. 7:728–735.
6. Blobel, C.P., and J.M. White. 1992. Structure, function and evolutionary relationship of proteins containing a disintegrin domain. Curr. Opin. Cell Biol. 4:760–765.
7. Blobel, C.P., D.G. Myles, P. Primakoff, and J.W. White. 1990. Proteolytic processing of a protein involved in sperm-egg fusion correlates with acquisition of fertilization competence. J. Cell Biol. 111:69–78.
8. Blobel, C.P., T.G. Wolfsberg, C.W. Turck, D.G. Myles, P. Primakoff, and J.M. White. 1992. A potential fusion peptide and an integrin ligand domain in a protein active in sperm-egg fusion. Nature (Lond.). 356:248–252.
9. Chomczynski, P., and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-cholroform extraction. Anal. Biochem. 162:156–159.
10. Creemer, J.W.M., R.J. Siezen, A.J.M. Roebroek, T.A.Y. Ayoubi, D. Huylebroeck, and W.J.M. Van de Ven. 1993. Modulation of furin-mediated proprotein processing activity by site directed mutagenesis. J. Biol. Chem. 268:21826–21834.
11. Emi, M., T. Katagiri, Y. Harada, H. Saito, J. Inazawa, I. Ito, F. Kasumi, and Y. Nakamura. 1993. A novel metalloprotease/disintegrin-like gene at 17q21.3 is somatically rearranged in two primary breast cancers. Nature Genet. 5:151–157.
12. Feng, S., J.K. Chen, H. Yu, J. Simon, and S. Schreiber. 1994. Two binding orientations for peptides to the Src SH3 domain: development of a general model for SH3-ligand interactions. Science (Wash. DC). 266:1241–1247.
13. Fuller, R.S., A.J. Brake, and J. Thorner. 1989. Intracellular targeting and structural conservation of a prohormone processing protease. Science (Wash. DC.) 246:482–486.
14. Gould, R.J., M.A. Polokoff, P.A. Friedman, T.F. Huang, J.C. Holt, J.J. Cook, and S. Niewiarowski. 1990. Disintegrins: a family of integrin inhibitory proteins from viper venoms. Proc. Soc. Exp. Biol. Med. 195:168–171.
15. Harlow, E., and D. Lane. 1988. Antibodies, a Laboratory Manual. Cold Spring Harbor Laboratories, Cold Spring Harbor, NY. pp. 53–138.
16. Heinlein, U.A.O., S. Wallat, A. Senftleben, and L. Lemaire. 1994. Male germ cell-expressed mouse gene TAZ83 encodes a putative, cysteine rich transmembrane protein (cyritestin) sharing homologies with snake venom

toxins and sperm egg fusion proteins. *Dev. Growth Diff.* 36:49–58.

17. Huang, T.F., J.C. Holt, E.P. Kirby, and S. Niewiarowski. 1989. Trigramin: primary structure and its inhibition of von Willebrand factor binding to glycoprotein IIb/IIIa complex on human platelets. *Biochemistry.* 28:661–666.

18. Huang, T.F., J.C. Holt, H. Lukasiewicz, and S. Niewiarowski. 1987. Trigramin: a low molecular weight peptide inhibiting fibrinogen interaction with glycoprotein IIb-IIIa complex receptors expressed on platelets. *J. Biol. Chem.* 262:16157–16163.

19. Hynes, R.O. 1987. Integrins: a family of cell surface receptors. *Cell.* 48:549–554.

20. Hynes, R.O. 1992. Integrins: versatility, modulation, and signaling in cell adhesion. *Cell.* 69:11–25.

21. Katagiri, T., Y. Harada, M. Emi, and Y. Nakamura. 1995. Human metalloprotease/disintegrin-like (MDC) gene: exon-intron organization and alternative splicing. *Cytogenet. Cell Genet.* 68:39–44.

22. Kemble, G.W., D.L. Bodian, J. Rose, I.A. Wilson, and J.M. White. 1992. Intermonomer disulfide bonds impair the fusion activity of influenza virus hemagglutinin. *J. Virol.* 66:4940–4950.

23. Kini, R.M., and H.J. Evans. 1992. Structural domains in venom proteins: evidence that metalloproteinases and nonenzymatic platelet aggregation inhibitors (disintegrins) from snake venoms are derived by proteolysis from a common precursor. *Toxicon.* 30:1–29.

24. Krätzschmar, J., B. Haendler, G. Langer, W. Boidol, P. Bringmann, A. Alagon, P. Donner, and W.D. Schleuning. 1991. The plasminogen activator family from the salivary gland of the vampire bat Desmodus rotundus: cloning and expression. *Gene (Amst.).* 105:229–237.

25. Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680–685.

26. Lim, W.A., F.M. Richards, and R. Fox. 1994. Structural determinants of peptide binding orientation and of sequence specificity in SH3 domains. *Nature (Lond.).* 372:375–379.

27. Mayer, B.J., and M.J. Eck. 1995. Minding your p's and q's. *Curr. Biol.* 4: 364–367.

28. Musial, J., S. Niewiarowski, B. Rucinski, G.J. Stewart, J.J. Cook, J.A. Williams, and L.H. Edmunds. 1990. Inhibition of platelet adhesion to surfaces of extracorporeal circuits by disintegrins. *Circulation.* 82:261–273.

29. Myles, D.G., L.H. Kimmel, C.P. Blobel, J.M. White, and P. Primakoff. 1994. Identification of a binding site in the disintegrin domain of fertilin required for sperm-egg fusion. *Proc. Natl. Acad. Sci. USA.* 91:4195–4198.

30. Pawson, T. 1995. Protein modules and signalling networks. *Nature (Lond.).* 373:573–580.

31. Pei, D., and S.J. Weiss. 1995. Furin-dependent intracellular activation of the human stromelysin-3 zymogen. *Nature (Lond.).* 375:244–247.

32. Perry, A.C.F., H.L. Barker, R. Jones, and L. Hall. 1994. Genetic evidence for an additional member of the metalloproteinase-like, cysteine rich (MDC) family of mammalian proteins and its abundant expression in the testis. *Biochem. Biophys. Acta.* 1207:134–137.

33. Perry, A.C.F., P.M. Gichuhi, R. Jones, and L. Hall. 1995. Cloning and analysis of monkey fertilin reveals novel a subunit isoforms. *Biochem. J.* 307: 843–850.

34. Perry, A.C.F., R. Jones, P.J. Barker, and L. Hall. 1992. A mammalian epididymal protein with remarkable sequence similarity to snake venom haemorrhagic peptides. *Biochem. J.* 286:671–675.

35. Primakoff, P., H. Hyatt, and J. Tredick-Kline. 1987. Identification and purification of a sperm surface protein with a potential role in sperm-egg membrane fusion. *J. Cell Biol.* 104:141–149.

36. Ruoslahti, E. 1991. Integrins. *J. Clin. Invest.* 87(1):1–5.

37. Scarborough, R.M., J.W. Rose, M.A. Naughton, D.R. Phillips, L. Nannizzi, A. Arfsten, A.M. Campbell, and I.F. Charo. 1993. Characterization of the integrin specificities of disintegrins isolated from American Pit Viper Venoms. *J. Biol. Chem.* 268:1058–1065.

38. Sparks, A.B., L.A. Quilliam, J.M. Thorn, C.J. Der, and B.K. Kay. 1994. Identification and characterization of Src SH3 ligands from phage displayed random peptide libraries. *J. Biol. Chem.* 269:23853–23856.

39. von Heijne, G. 1986. A new method for predicting signal sequence cleavage sites. *Nucleic Acids Res.* 14:4683–4690.

40. Watanabe, T., T. Nakagawa, J. Ikemizu, M. Nagahama, K. Murakami, and K. Nakayama. 1992. Sequence requirements for precursor cleavage within the constitutive secretory pathway. *J. Biol. Chem.* 267:8270–8274.

41. Weskamp, G., and C.P. Blobel. 1994. A family of cellular proteins related to snake venom disintegrins. *Proc. Natl. Acad. Sci. USA.* 91:2748–2751.

42. Weskamp, G., and L.F. Reichardt. 1991. Evidence that biological activity of NGF is mediated through a novel subclass of high affinity receptors. *Neuron.* 6:649–663.

43. White, J.M. 1992. Membrane fusion. *Science (Wash. DC).* 258:917–924.

44. Wolfsberg, T.G., J.F. Bazan, C.P. Blobel, D.G. Myles, P. Primakoff, and J.M. White. 1993. The precursor region of a protein active in sperm-egg fusion contains a metalloprotease and a disintegrin domain: structural, functional and evolutionary implications. *Proc. Natl. Acad. Sci. USA.* 90: 10783–10787.

45. Wolfsberg, T.G., P. Primakoff, D.G. Myles, and J.M. White. 1995. ADAM, a novel family of membrane proteins containing a disintegrin and metalloprotease domain: multipotential functions in cell–cell and cell–matrix interactions. *J. Cell Biol.* 131:1–4.

46. Wolfsberg, T.G., P.D. Straight, R.L. Gerena, A.-P.J. Huovila, P. Primakoff, D.G. Myles, and J.M. White. 1995. ADAM, a widely distributed and developmentally regulated gene family encoding membrane proteins with a disintegrin domain and a metalloprotease domain. *Dev. Biol.* 169:378–383.

47. Yaffe, D., and O. Saxel. 1977. Serial passaging and differentiation of myogenic cells isolated from dystrophic mouse muscle. *Nature (Lond.).* 270: 725–727.

48. Yagami-Hiromasa, T., T. Sato, T. Kurisaki, K. Kamijo, Y. Nabeshima, and A. Fujisawa-Sehara. 1995. A metalloprotease-disintegrin participating in myoblast fusion. *Nature (Lond.).* 377:652–656.

49. Yoshida, S., M. Setoguchi, Y. Higuchi, S. Akizuki, and S. Yamamoto. 1990. Molecular cloning of cDNA encoding MS2 antigen, a novel cell surface antigen strongly expressed in murine monocytic lineage. *Int. Immunol.* 2: 586–591.