Studies to clone a cell-surface DNA-binding protein involved in the binding and internalization of extracellular DNA have led to the isolation of a gene for a membrane-associated nucleic acid-binding protein (MNAB). The full-length cDNA is 4.3 kilobases with an open reading frame of 3576 base pairs encoding a protein of ~130 kDa (GenBank accession numbers AF255303 and AF255304). The MNAB gene is on human chromosome 9 with wide expression in normal tissues and tumor cells. A C3HC4 RING finger and a CCCH zinc finger have been identified in the amino-terminal half of the protein. MNAB bound DNA ($K_D \sim 4$ nM) and mutagenesis of a single conserved amino acid in the zinc finger reduced DNA binding by 50%. A potential transmembrane domain exists near the carboxyl terminus. Antibodies against the amino-terminal half of the protein immunoprecipitated a protein of molecular mass ~150 kDa and reacted with cell surfaces. The MNAB protein is membrane-associated and primarily localized to the perinuclear space, probably to the endoplasmic reticulum or trans-Golgi network. Characterization of the MNAB protein as a cell-surface DNA-binding protein, critical in binding and internalization of extracellular DNA, awaits confirmation of its localization to cell surfaces.

The interaction of extracellular DNA with the plasma membrane, and its subsequent internalization into the cell, can trigger specific cellular events. For instance, gene therapy involves the cellular binding and uptake of DNA as an intact molecule, with subsequent overexpression of the gene product(s). In some cases this overexpression can result in the stimulation of host immune responses as seen with DNA vaccines (1–3). Recently, there has been evidence demonstrating the immunostimulatory activity of bacterial DNA. The binding and internalization of bacterial DNA can initiate a variety of responses, both harmful as well as beneficial. Bacterial DNA has been reported to induce potentially harmful inflammation in the lung (4–8) and to activate macrophages with the subsequent release of tumor necrosis factor-$\alpha$ and interleukin-1$\alpha$ (9). In contrast, the immunostimulatory motif found in bacterial DNA (CpG) has been shown to mediate a number of beneficial effects. Hartman and colleagues (10) have shown that CpG motifs can activate human dendritic cells, implying a possible role in dendritic cell-mediated immune responses. In addition, the role of CpG motifs as adjuvants has recently been reported (11, 12). Binding and internalization of exogenous DNA are both required for initiation of these events.

The mechanism(s) that mediate the cellular binding and internalization of exogenous DNA remains, for the most part, undefined at the molecular level. There are numerous reports of the association of different forms of nucleic acids, such as DNA, RNA, and oligonucleotides, with cell surfaces (13–19) as well as reports of cellular binding and internalization of nucleic acids (20–26) to a variety of cell types. Oligonucleotides are bound and internalized by cells (19, 27). Whether oligonucleotides are internalized via a receptor-mediated endocytic pathway, a pinocytic mechanism, or a combination of both has not been resolved. Loke et al. (28) reported the uptake of unmodified oligonucleotides was a saturable phenomenon that was competitively inhibited by unmodified oligonucleotide, double or single stranded DNA, unrelated oligonucleotides, or RNA, suggesting a receptor-mediated endocytic pathway. A possible endocytic pathway for oligonucleotide uptake was described by Yakubov et al. (29) but it appeared that pinocytosis was the preferred pathway at a high concentration of oligonucleotides. Recently, the involvement of the macrophage-1 cell-surface molecule as a receptor for oligonucleotide binding has been reported (30). However, whether binding of oligonucleotides to macrophage-1 is the relevant mechanism by which DNA activates cells is unknown. In addition, the mechanism(s) for oligo and/or plasmid DNA binding and internalization by cells lacking macrophage-1 has yet to be established. Other studies substantiate the notion that cells possess specific cell-surface proteins that mediate nucleic acid binding and internalization. Bennett et al. (31) provided evidence that cell surface binding of high molecular weight DNA by human leukocytes was mediated by membrane-associated proteins with subsequent uptake and degradation of internalized DNA. Emlen and colleagues (32), working with hepatic cells, have demonstrated saturable DNA binding, suggesting a receptor-mediated process and Kawabata and colleagues (33) showed rapid uptake of plasmid DNA by the liver. Takagi and colleagues (34) demonstrated plasmid DNA binding and internalization by murine peritoneal

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Macrophages. However, characterization of the receptor(s) which mediate the binding and internalization of plasmid DNA remains undefined at the molecular level.

The molecular characterization of the cell-surface receptor(s) involved in the binding and internalization of DNA will provide the basis for the potential regulation of DNA binding to cells and the selective enhancement or diminishment of the biological effects of DNA. To facilitate the identification of this cell-surface receptor(s), we have demonstrated that DNA binding to cell surfaces could be inhibited by pretreatment of cells with serum from patients with SLE1 (35–37). Inhibition of DNA binding to cells was shown to be antibody-mediated and to be distinct from anti-DNA antibodies. We used a serum that inhibited DNA binding to cell surfaces and reacted to cell-surface DNA-binding proteins (21) to screen a Agt11 cDNA expression library derived from human monocytes. A novel membrane-associated protein of molecular mass ~130 kDa that binds DNA was identified. The molecular characterization and DNA binding properties of this molecule are detailed in this report.

MATERIALS AND METHODS

Cloning of MNAB—Serum from a patient with SLE was used to screen a Agt11 cDNA expression library from lipopolysaccharide-stimulated MOLT-4 cells (CLONTECH). This serum was antigen positive, had been depleted of anti-DNA antibodies by multiple clonal washes of the column with 15 mM reduced glutathione (Sigma), 50 mM Tris-HCl, pH 8.0. Alternatively, the fusion protein was cleaved in situ with PreScission protease (Amersham Pharmacia Biotech) to release the MNAB peptide from the GST peptide essentially according to the manufacturer's instructions. The released peptide was desalted and concentrated (Centricron 30; Amicon, Beverly, MA), then incubated with glutathione-Sepharose to remove traces of GST or GST fusion proteins. GST fusion proteins used as controls in DNA binding assays (GST/CBD, GST/HisT1) were provided by Dr. Rowland Kwok, University of Michigan, Ann Arbor, MI. Proteins were stored at ~80 °C.

Expression Vector for an Epitope-tagged MNAB—A BamHI-HpaI cDNA fragment containing the coding sequence for amino acids 1–1190 (missing the two most carboxyl terminal amino acids) of the MNAB was subcloned into pTripFlu (constructed by Dr. John Epstein, University of Pennsylvania, Philadelphia, PA). This vector contains the coding sequence for an epitope tag from the influenza virus HA in trimethylene inserted immediately 3′ of the multicloning site in pcDNA3 which is in-frame with the inserted MNAB cDNA sequence.

Production of Antibodies to MNAB—Polyclonal antibodies were produced to the purified GST/MNAB peptide from which the GST fusion partner had been removed by site-specific proteolysis. Female New Zealand White rabbits (2.3–3.0 kg) were injected subcutaneously with 60 μg of purified MNAB-(1–575) peptide emulsified 1:1 with TiterMax (CytRx Corp., Norcross, GA) in a final volume of 0.5 ml. The rabbits were boosted 4 weeks later with 15 μg of antigen/Titer-Max mixture, again 2 weeks later, and were maintained on a monthly intramuscular injection schedule thereafter. The final 2 injections consisted of a 3–5-μg antigen challenge. The rabbits were bled 7–10 days after each immunization and the sera analyzed for reactivity to the immunizing antigen. Sera were collected from each of the rabbits prior to immunization with the MNAB peptide and stored at −20 °C. All immune sera reacted against the immunizing antigen as assessed by ELISA and Western blot analysis. The sera is referred to as anti-MNAB.

Other Antibodies—Antibody to the human transferrin receptor was provided by Dr. Caroline Enns, OHSU, Portland, OR. Monoclonal anti-α-tubulin and rabbit anti-GST antibody (Cappel) were used as controls.

Cell Culture and Transfections—Cells were routinely cultured in Dulbecco's modified Eagle's medium (Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 2 mM t-glutamine, 100 units/ml penicillin G, 100 μg/ml streptomycin. Cells were stably expressing HA-tagged MNAB were established by transfection with pTripFlu-MNAB using LipofectAMINE (Life Technologies) and were selected with 500 μg/ml G418 (Life Technologies). G418-resistant cells were cloned by limiting dilution and tested for expression of HA-tagged MNAB by Western blotting.

Western Analysis—Lysates of cells were prepared on ice in lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) plus a protease inhibitor mixture (Sigma P8340, used at 1:100). Lysates were cleared by centrifugation at 20,000 × g, and the protein contents were estimated by Coomassie dye binding (Bio-Rad). Lysates were stored at −80 °C. Cells were fractionated into cytoplasmic and crude membrane fractions by Dounce homogenization on ice in hypotonic buffer (10 mM HEPES, pH 7.5, 5 mM KCl, 1.5 mM MgCl2, protease inhibitor mixture), followed by sequential centrifugation at 10,000 × g for 20 min then 100,000 × g for 60 min at 4 °C. The supernatant and pellet from the latter centrifugation were considered cytoplasmic and membrane fractions, respectively. Lysates (100 μg) and immunoprecipitates were heated at 85 °C in 2 × sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol)

1 The abbreviations used are: SLE, systemic lupus erythematosus; MNAB, membrane-associated nucleic acid-binding protein; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; GST, glutathione S-transferase; HA, hemagglutinin; ELISA, enzyme-linked immunosorbent assay; PAGE, polyacrylamide gel electrophoresis; bp, base pairs; EST, expressed tag sequence; TRAF, tumor necrosis factor receptor-associated factor.

2 D. Siess, C. T. Vedder, L. S. Merkins, T. Tanaka, A. C. Freed, S. L. McCoy, M. C. Heinrich, M. E. DeFeebach, R. M. Bennett, and S. H. Hefeneider, unpublished observations.
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for 10 min, separated by 7% SDS-polyacrylamide gel electrophoresis and electrophotically transferred to nitrocellulose (Hybond ECL, Amersham Pharmacia Biotech). The membranes were stained with Ponceau S to confirm transfer and identify standards. Membranes were blocked for 1 h at room temperature in 5% nonfat dry milk in TNT (50 mM Tris, pH 7.4, 100 mM NaCl, 0.05% Tween 20) at room temperature. The wells were washed and incubated sequentially for 1–2 h in primary antibody diluted in 5% nonfat dry milk in TNT followed by horseradish peroxidase-conjugated secondary antibody (Roche Molecular Biochemicals) in TNT. They were washed extensively in TNT in between each reagent. Antibody binding was detected with ECL and exposure of the membrane to Hyperfilm ECL (Amersham).

**Immunoprecipitations**—Lysates were precleared by incubating with protein A-agarose (Roche Molecular Biochemicals) for 3 h at 4 °C with rotation. Immunoprecipitations were carried out by incubating the pre-cleared lysates with either rabbit anti-MNAB or preimmune serum at 1:500, or mouse monoclonal anti-HA (2A25, Roche Molecular Biochemicals) or control mouse monoclonal antibody (MOPC141, Sigma) at 5 μg/reaction for 2 h at 4 °C with rotation. After incubation with protein A-agarose for 1 h with rotation at 4 °C, the immune complexes were collected by brief centrifugation then washed 3–4 times with lysis buffer. The immune complexes were then heated at 85 °C for 10 min in 25 μl of 2 × sample buffer.

**Immunofluorescence**—Cells (2 × 10⁶) were seeded into the wells of polylysine-coated 8-well Permanox chamber slides (Nunc, Naperville, IL) and incubated at 37 °C in 5% CO₂ for 2 days at room temperature. After 1 h at room temperature the wells were washed with PBS containing immobilized calf thymus DNA (United Biotech, Mountain View, CA). After 1 h at room temperature, the cells were fixed with 4% paraformaldehyde in PBS containing 1 mM each CaCl₂ and MgCl₂ (Ca,Mg-PBS) for 30 min at room temperature, washed twice with Ca,Mg-PBS, then permeabilized by incubation in permeabilization buffer (Ca,Mg-PBS, 10% fetal bovine serum, 0.05% sodium azide, and 0.1% Triton X-100) for 30 min at room temperature. The cells were incubated sequentially for 1 h at room temperature with primary antibody (1:100 rabbit anti-MNAB, 1:100 sheep anti-transferrin receptor, 1:200 mouse anti-γ-tubulin) then with the appropriate fluorophore-conjugated secondary antibody (1:200 Cy3 anti-rabbit IgG, 1:50 DTAF anti-mouse IgG (Accurate Chemical, Westbury, NY), or 1:100 fluorescein isothiocyanate anti-goat IgG (Tago, BIOSOURCE International, Camarillo, CA), diluted in permeabilization buffer, with extensive washing in permeabilization buffer following each antibody incubation. Slides were mounted in Antifade Light (Molecular Probes, Eugene, OR) and viewed with epifluorescence microscopy. Digital images were obtained with a Zeiss Axialphot Photo 3 fluorescence microscope equipped with a Hamamatsu digital charged coupled device camera and processed with QED imaging software. Non-immune sera from the appropriate species were used as controls for the primary antibodies. For doubly stained samples, control staining showed that there was no reactivity between the secondary antibody and the inappropriate primary antibody.

**Flow Cytometric Analysis**—Human 293 cells were lifted with 8 mM EDTA in PBS and washed once in PBS. Cells (5 × 10⁶) were incubated at 4 °C for 30 min with preimmune serum or anti-MNAB diluted in assay medium (1% fetal calf serum, 1 mM MgCl₂, 1 mM CaCl₂, and 0.1% sodium azide in PBS). After washing, cells were incubated at 4 °C for 30 min with fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (Caltag, Burlingame, CA) diluted 1:400 in assay medium. Viable cells were identified by incubation at 4 °C for 20 min with 2.5 μg/ml 7-Aminoactinomycin D (Molecular Probes) in assay medium. The fluorescence intensity of the cells was analyzed by FACScan (Becton Dickinson, Mountain View, CA) with CellQuest software. Ten thousand viable cells were counted in each analysis to determine the geometric mean fluorescence intensity. Means were compared with ANOVA and Tukey’s post-hoc multiple comparisons test.

**Site-directed Mutagenesis**—Mutagenesis of the zinc finger motif found in MNAB was done in pGEX-MNAB using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The coding sequences of the complimentary mutagenic primer pairs (Operon Technologies, Alameda, CA) used were: C416A, 5′-AAATACTAGTGAGATGCCGAGATTCCGACGAGC-3′; C416S, 5′-AAATACTAGTGAGATGCCTGAGATTCCGACGAGCAGC-3′; C431A, 5′-GTCCACGAGGACAACTGCTTACATGGCATCCGACCCTTCTTCCAGGGAGAATTCTGATCTGATCCGGCCATCCC-3′; C431S, 5′-GTCCACGAGGACAACTGCTTACATGGCATCCGACCCTTCTTCCAGGGAGAATTCTGATCTGATCCGGCCACCCC-3′. Altered nucleotides were bold and underlined. All mutations were verified by DNA sequencing.

**ELISA for DNA Binding**—Purified GST or GST fusion proteins were diluted in ELISA buffer (PBS, 1% bovine serum albumin) to 1 μg/ml and 100 μl of diluted proteins were added to wells of a microtiter plate containing immobilized calf thymus DNA (United Biotech, Mountain View, CA). After 1 h at room temperature the wells were washed with wash buffer (PBS containing 0.1% bovine serum albumin) and 100 μl of a 1:100 dilution of rabbit anti-GST (Sigma) in ELISA buffer were added. After 1 h at room temperature the wells were washed and 100 μl of a 1:1000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG (Roche Molecular Biochemicals) in ELISA buffer were added. After 1 h at room temperature the wells were washed and 100 μl of chromogenic substrate (3,3′,5′-tetramethyl benzidine, H₂O₂, Genzyme Diagnostics, San Carlos, CA) were added. Chromomogen conversion was measured by absorbance at 450 nm in a microplate reader (Bio-Tek Instruments, Winooski, VT).

**Nitrocellulose Filter Assay for DNA Binding**—Quigen (Valencia, CA) control was labeled with Bio-Tag (35S) nucleotides (100 μCi/ml) and linearized with BglII restriction enzyme and sequentially extracted with phenol:chloroform:isoamyl alcohol (24:24:1), chloroform:isoamyl alcohol (24:1) then ethanol precipitated. After re-suspension the DNA was labeled by a fill-in reaction with the Klenow fragment of DNA polymerase I and [α-32P]dATP (800 or 3000 Ci/mmol; NEN Life Sciences, Boston, MA). Unincorporated label was removed by spin chromatography through Sephadex G-50. The specific activity of the labeled DNA was determined by precipitation with 10% trichloroacetic acid and scintillation counting. For the binding assay, the GST/ MNAB protein and DNA were diluted into binding buffer (20 mM HEPS, pH 7.4, 75 mM NaCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5% glycerol, 100 μg/ml bovine serum albumin) to make 2 × stocks. The GST/MNAB protein and DNA were combined and incubated for 30 min at 30 min at room temperature. The sample buffer.

**RESULTS**

**Cloning Strategy**—One million plaques from a λgt11 lipopolysaccharide-activated human monocye cDNA library were screened with an SLE serum that was previously shown to inhibit DNA binding to cell surfaces (21). Ten clones were identified and plaque purified. Sequence analysis of the 1.4-kb clone 88, which was highly reactive on Western blots with the SLE serum, revealed an open reading frame coding for a partial protein. The reading frame remained open at the 5′ end and a stop codon was found near the 3′ end of the clone. Thus, clone 88 coded for the 3′ end of a larger protein.

Biotinylated DNA was used to isolate DNA-binding proteins from the S49 murine cell line. A 150-kDa protein was obtained that reacted by Western blot with both the SLE serum used to screen the library and with a rabbit polyclonal antibody that was raised against the fusion protein encoded by clone 88. The reading frame remained open at the 5′ end and a stop codon was found near the 3′ end of the clone. Thus, clone 88 coded for the 3′ end of a larger protein.

**Gene Expression and Protein Sequence Analysis** — A survey of MNAB gene expression by Northern blot analysis revealed two major transcripts of 9.5 and 6.8 kb in all human tissues and cancer cell lines examined (Fig. 1). Several smaller transcript sizes were also observed in some of those tissues and cell lines. Expression was most abundant in spleen, testis, parathyroid, and HeLa cell lines. An homology search using MNAB sequence identified the genomic sequence which was known to be located at q34 on chromosome 9 (GenBank accession number 007066, marker HM9.89 on Contig CHR9.SL.27). The genomic clone sequence, which covered 85% of the cDNA starting from the 5′ end, revealed the location of 16 complete exons. A Blast search of the expressed tag sequence (EST) data base indicated wide
expression of this gene in normal human tissue (liver/spleen, prostate epithelial, germinal B cell, white adipose, pregnant uterus, fetal heart/liver, and spleen) and in tumor and transformed cells (Jurkat, HL60, 293, G361, B-cell lymphotic leukemia, colon tumor, melanoma, and parathyroid tumor).

Hydropathy analysis (41) identified a 38-amino acid hydrophobic region near the carboxyl terminus of the protein (amino acids 1133–1171) which is a potential transmembrane domain. No consensus amino-terminal signal sequence was identified. A proline-rich region (20%) spanning amino acids 549–809 and 7 consensus sites for N-linked glycosylation at amino acid positions 122, 394, 430, 451, 466, 468, and 1150, have been identified (Fig. 2). The calculated isoelectric point of the MNAB protein is 6.4.

Blast search has also identified two motifs: a C3HC3D RING finger subtype located near the amino terminus (amino acids 14–50) and a CCCH zinc finger located near the center of the MNAB protein sequence (amino acids 416–435). An alignment of several RING finger motifs is shown in Fig. 3A. MNAB differs from the originally identified C3HC4 RING finger motif by the replacement of the last cysteine with an aspartic acid. The alignment of the conserved cysteines and histidines of the CCCH zinc finger motif is shown in Fig. 3B.

Characterization of MNAB Protein Expression in Mammalian Cells—Immunoprecipitation and Western blotting experiments with anti-MNAB antibodies identified a protein of M_0, ~1.5 × 10^6 in most cells (293, COS7, G361, Hela, HREC605, Molt-4, Raji, A549, and B16). A protein with this same (or slightly slower) mobility was also detected by immunoprecipitation and/or Western blotting with either rabbit anti-MNAB or mouse monoclonal anti-HA from lysates of cells (293-MNAB/flu) stably transfected with an expression vector for a carboxy-terminal HA-tagged MNAB (pMNAB/flu, Fig. 4).

Subcellular Protein Localization—Fractionation by centrifugation of Dounce-homogenized 293-MNAB/flu cells into soluble and crude membrane fractions followed by Western blotting with either anti-MNAB or anti-HA showed that essentially all the MNAB protein in those cells was associated with the membrane fraction (Fig. 5A). Indirect immunofluorescence on fixed, permeabilized cells (A549, Fig. 5B; COS7, HeLa) showed that anti-MNAB staining was predominantly localized to the perinuclear region of the cell. No nuclear staining was observed. Double staining with anti-MNAB and anti-transferrin receptor antibodies showed partial co-localization of the MNAB and transferrin receptor. The MNAB did not co-localize with the transferrin receptor in peripheral endosomes and neither MNAB nor transferrin receptor were visualized on cell surfaces suggesting the number of receptors on the cell surface are below the limits of detection in this assay. Double staining with anti-MNAB and anti-γ-tubulin showed no co-localization, indicating that MNAB does not localize to the centrosome. The observed staining pattern is consistent with the results obtained by cell fractionation and with the protein being located primarily in the ER or trans-Golgi network. However, these results do not exclude the possibility that the MNAB protein is expressed on cell surfaces.

To determine if MNAB is located on the cell surface, human kidney cell line 293 was incubated with anti-MNAB. Antibody binding was detected by flow cytometry with fluorescein isothiocyanate-labeled secondary antibodies to rabbit IgG. At all serum dilutions the fluorescence intensity of the cells incubated with anti-MNAB was significantly higher than that of cells incubated with preimmune serum (p < 0.003) (Fig. 6) suggesting that MNAB is expressed on the cell surface. However, attempts to overexpress the MNAB protein on the cell surface, as assessed by an increase in DNA or anti-MNAB antibody binding to the surface of MNAB-transfected cells, have not been successful.

Soluble MNAB Fusion Protein Binds DNA—Purified GST/MNAB fusion protein bound to ELISA plates coated with either calf thymus DNA (Fig. 7) or plasmid DNA. Negative controls using GST peptide alone or two irrelevant GST fusion proteins (GST/CBD, GST/HST.1) showed no binding. To demonstrate that the binding of DNA by the soluble form of the MNAB was not simply due to a charge related interaction, the role in DNA binding of the zinc finger domain at amino acids 416–435 was examined (see Fig. 2). Using site-directed mutagenesis, the codons for the conserved zinc finger cysteines at either amino acids 416 or 431 were altered to a codon for either alanine or serine. The mutagenized GST/MNAB fusion proteins were expressed in E. coli and affinity purified on glutathione-Sepharose, then tested for their ability to bind to immobilized DNA by ELISA. Mutagenesis of either cysteine 416 or 431 reduced DNA binding to approximately 50% of the level observed for wild type GST/MNAB fusion protein (Fig. 7). The affinity of GST/MNAB for DNA was estimated using a nitrocellulose filter binding assay (Fig. 8). GST/MNAB (2 nM) and labeled DNA (200 psi) were titrated with increasing amounts of unlabeled DNA (0–48 nM). A Scatchard transformation of the data yields a KD of 4 nM.

**DISCUSSION**

Evidence for high molecular weight DNA binding to cell surfaces, with subsequent internalization via a receptor-mediated mechanism, was first reported in 1985 by Bennett et al. (31). In an attempt to identify, at the molecular level, a cell-surface receptor(s) that binds nucleic acids we have cloned a novel human gene coding for a membrane-associated protein (MNAB) that binds DNA. The deduced amino acid sequence of this novel DNA-binding protein has several distinctive features, including a RING finger near the amino-terminal, an internal zinc finger, multiple possible glycosylation sites and a...
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A C3HC3D RING Finger Homologies

| MNB | H. sapiens ARD1 | TEFLGLCPITC-----YNEFDFRNVKPIISLGCHTVCNLKNIKRA----CFPDQTAIN | 58 |
| C. elegans cDNA EST 3879246 | VKLKLYCVDVPSL---QDRNVRLLLGCHTVCNLKNIKRA----CFPDQTAIN | 58 |
| C. elegans 5.8K protein | KRLKLCPLKGMPFVPGTST---QGRFEDTCDPLSBE--YVFKPDEQDPLD | 62 |
| C. elegans cDNA EST 3878719 | DEQLICITCOSVLEEPSVPGAR---ADRAFWCKTOMKFPGO--Q---TCEFVDRUVT | 61 |

C3HC3D motif

| MNB | C. elegans PIE-1 | ETMQPOPQNSKYSMTSCMDLRQOOGSCPRGNTCTPFABSQKLEKYYALKN | 448 |
| C. elegans TIS11B | HTEYXTRLCAFDFRGGCMPEGYCNHNYCTAYQHODTELQPRKOS | 136 |
| S. cerevisiae CHI1 | QPOLQNLKLYTELQCSPKTCGYCYNKQPCAMBNEKLK---C---C---H--- | 235 |

CCCH motif

| MNB | C. elegans PIE-1 | YKTELC-----C---C---H--- | 166 |
| C. elegans DTIS 11 | SSRTYKTELCFPCRENGCITYGKQMFAGHSHIEL | 181 |
| H. sapiens TIS11B | QPOLQNLKLYTELQCSPKTCGYCYNKQPCAMBNEKLK---C---C---H--- | 235 |

FIG. 3. A, alignment of conserved cysteines, histidines, and aspartic acids of the C3HC3D RING finger. Homo sapiens ARD1 GTP-binding protein (GenBank A46054) (66), H. sapiens CART1 protein (GenBank X80200) (67), H. sapiens SBBIO3 putative protein (GenBank 5032071), C. elegans cDNA EST (GenBank 3879246), C. elegans hypothetical 25.8 kDa protein (GenBank 2496825), C. elegans cDNA EST (GenBank 3878739). B, alignment of conserved cysteines and histidines of the CCCH type zinc finger. C. elegans PIE-1 (GenBank U62896), Drosophila melanogaster DTIS 11 (GenBank U13397), H. sapiens TIS11B Buryrate response factors (EFT Response factor) (GenBank X79068), Saccharomyces cerevisiae CHI1 zinc finger protein (GenBank L42133).

B C3H Zinc Finger Homologies

| MNB | C. elegans PIE-1 | ETPQPOPQNSKYSMTSCMDLRQOOGSCPRGNTCTPFABSQKLEKYYALKN | 448 |
| D. melanogaster DTIS 11 | HTEYXTRLCAFDFRGGCMPEGYCNHNYCTAYQHODTELQPRKOS | 136 |
| C. elegans TIS11B | QPOLQNLKLYTELQCSPKTCGYCYNKQPCAMBNEKLK---C---C---H--- | 235 |

FIG. 4. Analysis of MNAB protein expression in mammalian cells. Immunoprecipitation of MNAB and HA-tagged MNAB proteins in stably transfected 293-MNAB/flu cells. Lane 1, lysate of 293 cells; lane 2, lysate of 293-MNAB/flu cells; lanes 3–6, immunoprecipitation of 293-MNAB/flu cell lysates with: rabbit preimmune serum ([anti-MNAB (lane 4) IgH (lane 2) anti-HA (lane 6)]). Detected by Western blotting with rabbit anti-MNAB. β-Actin, immunoprecipitating rabbit IgG heavy chain.

Fig. 3. A, alignment of conserved cysteines, histidines, and aspartic acids of the C3HC3D RING finger. Homo sapiens ARD1 GTP-binding protein (GenBank A46054) (66), H. sapiens CART1 protein (GenBank X80200) (67), H. sapiens SBBIO3 putative protein (GenBank 5032071), C. elegans cDNA EST (GenBank 3879246), C. elegans hypothetical 25.8 kDa protein (GenBank 2496825), C. elegans cDNA EST (GenBank 3878739). B, alignment of conserved cysteines and histidines of the CCCH type zinc finger. C. elegans PIE-1 (GenBank U62896), Drosophila melanogaster DTIS 11 (GenBank U13397), H. sapiens TIS11B Buryrate response factors (EFT Response factor) (GenBank X79068), Saccharomyces cerevisiae CHI1 zinc finger protein (GenBank L42133).

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A C3HC3D RING Finger Homologies

| MNB | H. sapiens ARD1 | TEFLGLCPITC-----YNEFDFRNVKPIISLGCHTVCNLKNIKRA----CFPDQTAIN | 58 |
| C. elegans cDNA EST 3879246 | VKLKLYCVDVPSL---QDRNVRLLLGCHTVCNLKNIKRA----CFPDQTAIN | 58 |
| C. elegans 5.8K protein | KRLKLCPLKGMPFVPGTST---QGRFEDTCDPLSBE--YVFKPDEQDPLD | 62 |
| C. elegans cDNA EST 3878719 | DEQLICITCOSVLEEPSVPGAR---ADRAFWCKTOMKFPGO--Q---TCEFVDRUVT | 61 |

C3HC3D motif

| MNB | C. elegans PIE-1 | ETMQPOPQNSKYSMTSCMDLRQOOGSCPRGNTCTPFABSQKLEKYYALKN | 448 |
| C. elegans TIS11B | HTEYXTRLCAFDFRGGCMPEGYCNHNYCTAYQHODTELQPRKOS | 136 |
| S. cerevisiae CHI1 | QPOLQNLKLYTELQCSPKTCGYCYNKQPCAMBNEKLK---C---C---H--- | 235 |

CCCH motif

| MNB | C. elegans PIE-1 | YKTELC-----C---C---H--- | 166 |
| C. elegans DTIS 11 | SSRTYKTELCFPCRENGCITYGKQMFAGHSHIEL | 181 |
| H. sapiens TIS11B | QPOLQNLKLYTELQCSPKTCGYCYNKQPCAMBNEKLK---C---C---H--- | 235 |

FIG. 3. A, alignment of conserved cysteines, histidines, and aspartic acids of the C3HC3D RING finger. Homo sapiens ARD1 GTP-binding protein (GenBank A46054) (66), H. sapiens CART1 protein (GenBank X80200) (67), H. sapiens SBBIO3 putative protein (GenBank 5032071), C. elegans cDNA EST (GenBank 3879246), C. elegans hypothetical 25.8 kDa protein (GenBank 2496825), C. elegans cDNA EST (GenBank 3878739). B, alignment of conserved cysteines and histidines of the CCCH type zinc finger. C. elegans PIE-1 (GenBank U62896), Drosophila melanogaster DTIS 11 (GenBank U13397), H. sapiens TIS11B Buryrate response factors (EFT Response factor) (GenBank X79068), Saccharomyces cerevisiae CHI1 zinc finger protein (GenBank L42133).

The MNAB gene was found to be widely expressed in human tissues and is conserved across several species. The most abundant transcripts were shown by Northern blot analysis to be in the spleen, testis, ovary, and small intestines. MNAB gene expression was also observed in all tumor and transformed cell lines examined. A search of the mouse EST data base indicated potential transmembrane domain near the carboxyl-terminal of the protein.

The MNAB gene was found to be widely expressed in human tissues and is conserved across several species. The most abundant transcripts were shown by Northern blot analysis to be in the spleen, testis, ovary, and small intestines. MNAB gene expression was also observed in all tumor and transformed cell lines examined. A search of the mouse EST data base indicated conservation of the sequence in the mouse homologue as well. Several mouse clones were identified that have 76–95% identity with the MNAB gene clone from HL60 cells revealed a divergent sequence at its 3' end lacking the entire putative transmembrane domain.2 Since HL60 cells have been found to have a low DNA binding capacity (<5% by FACS)2 (45), it may be possible that the MNAB gene variant found in HL60 cells, which lacks the putative transmembrane domain, codes for a secreted soluble form of the receptor which is not found on cell surfaces, as has been observed for other receptors (44, 46–49).

Although, the function of the RING finger domain in the MNAB protein is not yet characterized, it could be involved in nucleic acid binding. Recently it was shown that the RING-
finger motif in the proto-oncogene MDM2 specifically binds RNA (50). The consensus RING finger motif is described by the sequence C-x_2-C-x_9-39-C-x_1-3-H-x_2-3-C-x_4-48-C-x_2-C, termed C3HC4, but several variations have been described (51). The MNAB motif is of the subtype C3HC3D RING finger where the last cysteine is replaced by an aspartic acid. Other members of this subtype include CART1 (a member of the TRAF family, see below), ARD1 GPT-binding protein, various uncharacterized proteins from C. elegans, and SSBI03 an uncharacterized hypothetical human protein (Fig. 3A). No specific function has yet been attributed to this subtype of the motif.

There are several hundred cDNAs encoding RING finger proteins in GenBank (52), and several RING finger subfamilies have been identified based on the presence of multiple common motifs. For example, the RING-B box coiled-coil family consists of proteins with a RING finger and one or two zinc-binding domains (B-box motif) followed by a leucine coiled-coil domain (53–55). A different arrangement of motifs describes the TRAF family members which contain a RING finger domain, a large cysteine/histidine-rich region, a coiled-coil domain, and a tumor necrosis factor receptor-associated factor (TRAF) domain (56). A common functionality among subfamily members based on these groupings of motifs has been reported (51). The RING-B box coiled-coil family members are proto-oncogenes, whereas the TRAF family members are involved in signal transduction pathways. It is interesting to note that the TRAF-1 gene maps to q33–34 in close proximity to MNAB on chromosome 9 (57). Another family of RING fingers has been linked to vacuolar/peroxisome biogenesis (58). In some cases the RING finger-containing protein is part of a multiprotein complex (58, 59). Recently, the RING finger motifs of eight otherwise unrelated proteins have been shown to have ubiquitin ligase activity (52, 60). Modification with chains of ubiquitin constitutes the primary mechanism by which proteins are targeted for proteasomal degradation (61). These findings have led to the hypothesis that RING finger containing proteins may
regulate a wide variety of cellular activities, such as growth factor receptor signaling, cell cycle progression, and DNA repair by facilitating ubiquitin-dependent degradation of key components of these processes. Whether the RING finger motif in the MNAB protein mediates DNA binding and/or ubiquitination remains to be established.

The zinc finger in the MNAB protein may participate in DNA binding because mutagenesis of a single conserved amino acid in this domain significantly reduces DNA binding. However, it is possible that mutagenesis of a single amino acid resulted in a change in the confirmation of the MNAB protein, limiting its ability to effectively bind DNA. The MNAB motif is of the CCCH subfamily of zinc finger motifs which is comprised of the consensus sequence YKTEC-x$_5$-C-x$_7$-C-x$_8$-H usually found in tandem. The MNAB protein contains a single motif in which three (YKT) of the 5 amino acids (YKTEL) leading into the first cysteine are conserved (see Fig. 3). The function of this motif in the majority of subfamily members is largely uncharacterized, however, one other member of this subfamily, the zf$+^D$ protein, has been reported to bind DNA (62). Recently, tristetraprolin, the prototype of the CCCH subfamily, has been shown to bind RNA via its zinc finger. Tristetraprolin regulates expression of tumor necrosis factor-$\alpha$ by binding to the AU-rich element in the tumor necrosis factor-$\alpha$ mRNA targeting it for degradation (63, 64). Tristetraprolin also appears to regulate granulocyte macrophage-colony stimulating factor mRNA stability by a similar mechanism (65).

In summary, we have cloned a novel human gene coding for a membrane-associated protein. Given the wide expression of the MNAB gene, both within and between species, this protein most likely has a role in non-organ specific cellular processes. The MNAB protein binds DNA with relatively high affinity, suggesting that this may be an important function for this molecule. The demonstration that this membrane-associated DNA-binding protein is a cell-surface receptor critical in the binding and internalization of extracellular DNA awaits definitive confirmation of its location on the cell surface.

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