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Human Myeloid Cell Nuclear Differentiation Antigen Binds Specifically to Nucleolin

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Abstract The human myeloid cell nuclear differentiation antigen (MNDA) is a nuclear protein expressed specifically in cells of the myelomonocytic lineage and regulated by interferon α in a cell-specific fashion. MNDA is also a member of a family of interferon-regulated genes of unknown function. In an effort to elucidate the function of MNDA, three techniques (affinity purification, coimmunoprecipitation, and protein blot assay) were used to characterize its specific protein binding activities. Microsequence analysis showed that MNDA bound the 100 kDa nucleolin protein. The identification of nucleolin was confirmed by immunoreaction with specific antibodies. MNDA contains motifs which could account for specific binding to nucleolin. Nucleolin binds other macromolecules and exhibits features consistent with roles in signal transduction, production of ribosomes, nuclear matrix structure, and regulation of transcription. The present results indicate that the function of MNDA is most likely related to interactions with other proteins. Through these associations, MNDA could contribute cell/lineage- and differentiation-specific limits to the function of ubiquitous proteins such as nucleolin. Further analysis of MNDA protein binding could be critical to elucidating the function of MNDA and could contribute to understanding the function of the products of other members of this interferon-inducible family of genes. © 1995 Wiley-Liss, Inc.

Key words: nucleolus, monocyte, granulocyte, nuclear matrix, interferon

The human myeloid cell nuclear differentiation antigen (MNDA) is a nuclear protein expressed only in maturing cells of the myelomonocytic lineage and in myeloid cell leukemia blast cells that resemble the same stages of differentiation as the normal MNDA positive cells [Briggs et al., 1994b,c; Cousar and Briggs, 1990; Dawson et al., 1995; Goldberger et al., 1984, 1986]. MNDA-positive cells can be induced to differentiate along the monocyte/macrophage and/or granulocyte pathways [Briggs et al., 1994b]. The distribution of MNDA within the nucleus is affected by cell differentiation and DNA damage [Duhl et al., 1989]. MNDA expression is upregulated by interferon α specifically [Briggs et al., 1992, 1994c].

The relationship of MNDA to the human IFI 16 protein and the products of a cluster of mouse genes (gene *200 cluster*) is based on the presence of a 200 amino acid conserved region that is present in one or two copies in each member of the family, location of the gene in a conserved region on chromosome 1, and responsiveness to interferon-stimulated upregulation or induction of gene expression [Briggs et al., 1994a; Choubey et al., 1989; Dawson et al., 1995; Trapani et al., 1992]. While sharing many properties, there are also differences between MNDA and members of the gene family as well as between other members. In the case of the two human genes, *MNDA* is regulated specifically by interferon α while the *IFI 16* is responsive to interferon γ and less so to α (Dawson et al., 1995). *MNDA* and *IFI 16* are expressed constitutively in different lineages of hematopoietic cells (Dawson et al., 1995; Goldberger et al., 1986; Trapani et al., 1992], and only one of the characterized mouse genes has been reported to be cell-specific [Tannenbaum et al., 1993]. The determination of intracellular localization of some of the gene products also showed variability. MNDA and the IFI 16 localize throughout the nucleus [Dawson and Trapani, 1995; Goldberger et al., 1984, 1986], includ-

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ing the nucleolus, and the mouse 204 protein (one of the products of the six *200 cluster* genes) localizes primarily in the nucleolus [Choubey and Lengyel, 1992]. The mouse 202 protein is found in both the cytoplasm and nucleus and becomes concentrated in the nucleus following interferon treatment [Choubey and Lengyel, 1993]. The 202 protein is localized on chromosomes in mitotic cells, while MNDA becomes dispersed throughout the cell, excluding the mitotic chromosomes.

At the present time, the function of MNDA and the products of other members of this gene family are unknown. The present study demonstrates that the MNDA binds other proteins specifically, and its binding to nucleolin suggests that MNDA is involved in signal transduction and/or nuclear organization and structure. These data provide a molecular basis for the intranuclear distribution of at least one fraction of MNDA and indicate that future investigations of the products of this gene family will require consideration of protein-protein interactions.

MATERIALS AND METHODS

Cell Lines and Cellular Fractionation

MNDA expressing human promonocytic leukemia U937 and nonexpressing human erythroleukemic K562 cells were cultured as described previously [Goldberger et al., 1986]. HeLa cells were grown in DMEM (Dulbecco's Modified Eagle Media) (Life Technologies, Inc., Gaithersburg, MD) supplemented with 10% defined bovine calf serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, and antibiotics. Cells were hypotonically lysed [Burrus et al., 1992], and crude nuclei were pelleted by centrifugation (600*g*, 10 min). The supernatant was cleared by centrifugation (13,000*g*, 15 min) and designated as the cytosol fraction. The crude nuclei were further purified by separation over a one-step sucrose gradient, and nuclear protein extracts were prepared as described earlier [Burrus et al., 1992]. Protein concentration was determined by the method of Bradford [1976].

Synthesis of Recombinant MNDA Protein

To facilitate subcloning, synthetic DNA oligonucleotides (5-TCACGGTACCGAAGGTCGT-GTGAATGAATACAAGAAAATTCT-3 and 5-CAC-CAAGCTTTCATATTTCAATTAACATTC-3), containing 5′ noncomplementary extensions with inserted restriction sites (KpnI and HindIII) not present in the *MNDA* open reading frame (ORF) were used to PCR amplify the entire *MNDA* ORF minus the ATG initiation codon. A full-length *MNDA* cDNA previously described [Briggs et al., 1992] and subcloned in pBluescript (Stratagene, La Jolla, CA) was used as the template. The MNDA fragment was excised and ligated into the pQE30 expression vector (Qiagen, Chatsworth, CA) which fuses a 6× histidine tag 5′ to the amino terminus of the *MNDA* ORF. The correct inframe ligation was confirmed by sequencing through the linker region into the 5′-*MNDA* ORF. The constructed pQE30/*MNDA* was used to transform *E. coli* strain M15, which carries a repressor plasmid, pREP4. After isopropylthio-β-D-galactoside induction, the expressed recombinant MNDA (rMNDA) protein was purified from the bacterial lysate on a Ni^{2+}-NTA-agarose™ column (Qiagen) using the manufacturer's recommended procedure. Protein was quantified as described above.

Labeling of MNDA

Analysis of the MNDA amino acid sequence with the aid of the IntelliGenetics Suite Programs (IntelliGenetics, Inc., Mountain View, CA) revealed two consensus cAMP-dependent protein kinase phosphorylation sites corresponding to amino acids 135 (KRKT) and 334 (KKNT). The catalytic subunit of cAMP-dependent kinase from bovine heart (Sigma, St. Louis, MO) was obtained as a lyophilized powder and reconstituted with 40 mM dithiothreitol (DTT). Ten units of the reconstituted enzyme was added to 20 μg of rMNDA in 100 μl of reaction mixture containing 20 mM Tris-HCl, pH 7.5, 1 mM DTT, 150 mM NaCl, 12 mM $MgCl_2$, 1 mCi [γ-^{32}P]ATP (approximately 7,000 mCi/mmol). After 3–4 h incubation at 30°C, the labeled protein was separated from the free nucleotide by a Sephadex G-50 spun-column preequilibrated with 25 mM HEPES/KOH, pH 7.7, 12.5 mM $MgCl_2$, 20% glycerol, 100 mM KCl, 1 mM DTT, and 2 mg bovine serum albumin/milliliter.

Affinity Purification of MNDA Binding Proteins

Purified monoclonal antibody against MNDA (3C1) covalently coupled to agarose beads [Hudson et al., 1988] at approximately 15 mg of antibody per milliliter of beads was incubated (by rotation) with excess rMNDA in 10 mM Tris-HCl, pH 8.0, 0.35 M NaCl, 1 mM $MgCl_2$, 0.1

mM EGTA, 0.1% NP-40, and 10% glycerol for 18–24 h at 4°C. Nonbound rMNDA was removed by extensive washing with phosphate-buffered saline. The rMNDA antibody complex (rMNDA-Ab-bead) was then incubated with U937 nuclear extract for an additional 18–24 h in binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM $MgCl_2$, 0.1 mM EGTA, 0.1% NP-40, 10% glycerol) containing 100 mM NaCl in a final nuclear protein concentration of 0.5–0.8 mg/ml and 15 μl rMNDA-Ab-bead complexes/milliliter. After incubation, the rMNDA-Ab-beads were pelleted and extensively washed with 0.1 M NaCl binding buffer. The rMNDA-associated proteins were eluted by increasing the NaCl concentration to 0.5 M. Coimmunoprecipitation experiments were also completed whereby the monoclonal antibody beads (Ab-bead) were incubated directly with U937 or K562 cell nuclear protein extracts in the 0.1 M NaCl binding buffer as described above. Proteins that bound to the Ab-bead preparation were analyzed after extensive washing with binding buffer.

Analysis of Electrophoretically Separated Protein Samples

To perform the protein blotting assay, protein fractions were solubilized without heating and separated by 7.5% SDS-polyacrylamide gel electrophoresis as described by Laemmli [1970]. The separated proteins were electrotransferred to nitrocellulose membrane as described by Towbin et al. [1979] but without methanol in the transfer buffer. The immobilized proteins were denatured and renatured by passage through a graded series of buffers with decreasing guanidinium-HCl [Masson et al., 1993]. These renaturation steps were followed by incubation with [^{32}P]rMNDA, washing, and exposure to film essentially as described by Masson et al. [1993].

Immunoblotting procedures have been described previously [Goldberger et al., 1986]. Polyclonal rat or rabbit antiserum against rMNDA, rat monoclonal antibodies against natural MNDA [Hudson et al., 1988], or affinity purified rabbit polyclonal antibodies against hamster nucleolin peptide KKMAPPPKEVE (residues 14–24) prepared by standard procedures were used as primary antibodies. Alkaline phosphatase–conjugated second antibodies (Life Technologies Inc.) were used to develop the immunoblots. Coomassie brilliant blue dye was used to visualize proteins in SDS-polyacrylamide gels.

Amino Acid Sequencing of 100 kDa MNDA Binding Protein

The MNDA-associated nuclear proteins selectively isolated using rMNDA-Ab-bead complexes as described above were separated on a 7.5% SDS-polyacrylamide electrophoretic gel and transferred to polyvinylidene difluoride membrane (BioRad, Hercules, CA). After brief Ponceau S staining, the most prominent 100 kDa protein band was excised and submitted to Harvard Microchem (Cambridge, MA) for direct peptide sequencing.

RESULTS

Expression and Purification of rMNDA

Full-length rMNDA was expressed as a soluble protein and purified to homogeneity as assessed by SDS polyacrylamide electrophoresis (Fig. 1, lane 3). The authenticity of the rMNDA was confirmed by observing a molecular weight shift (an increase of 2.2 kDa over natural MNDA due to the addition of the histidine tag) when analyzed by denaturing polyacrylamide electrophoresis and by demonstrating reactivity with monoclonal antibodies prepared previously against natural MNDA [Hudson et al., 1988] (data not shown). The authenticity of the rMNDA was further confirmed through the demonstration that polyclonal antibodies raised against the rMNDA reacted specifically with the natural MNDA found in expressing cell lines (data not shown).

Analysis of rMNDA Binding Proteins in MNDA-Positive and -Negative Cells Using Affinity Purification

MNDA-Ab-bead complexes were incubated with U937 cell nuclear protein extract as described in the Materials and Methods. Eluted bound proteins consistently yielded one major band at 100 kDa (Fig. 1, upper arrow) and a number of minor bands (Fig. 1, lane 2). Similar bands including the 100 kDa MNDA binding protein were also bound to the rMNDA-Ab-bead complexes when nuclear protein extracts from the MNDA-negative cell line (K562) (data not shown) were tested, indicating that MNDA binds ubiquitous proteins.

Amino Acid Sequence Analysis of the 100 kDa rMNDA Binding Protein

Approximately 100 pmol of the 100 kDa protein prepared as described in Materials and

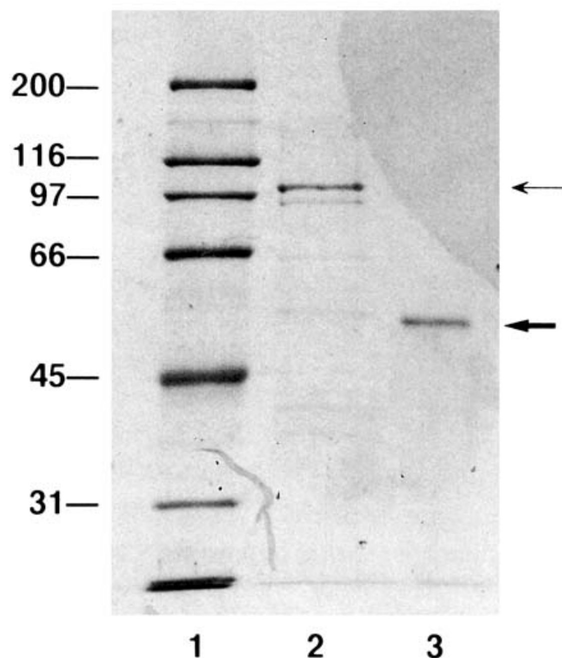

Fig. 1. SDS-polyacrylamide gel electrophoresis of purified rMNDA and affinity purified rMNDA binding proteins. A 7.5% polyacrylamide gel was stained with Coomassie brilliant blue. Proteins bound by rMNDA were isolated from U937 cell nuclear protein extract using rMNDA-Ab-bead complexes (*lane 2*). The upper arrow in the right margin locates the 100 kDa protein band. Purified rMNDA (*lane 3*) (lower arrow in right margin). *Lane 1:* Protein markers with molecular weights indicated (kDa).

Methods was submitted for peptide separation and microsequence analysis. Two well-separated fragments were sequenced. The first peptide provided a 14 amino acid sequence of GFG-FVDFNSEEDAK which is 100% identical to a sequence within human nucleolin. The sequence of the second peptide, TGISDVFAK, was also identical to a sequence within nucleolin. Additionally, the apparent molecular weight of this rMNDA binding protein observed in denaturing polyacrylamide electrophoresis was consistent with that reported for nucleolin [Olson, 1990].

Coimmunoprecipitation Analysis of the MNDA Protein Binding Activity

To confirm that natural MNDA binds nucleolin, the Ab-bead preparation (described in Materials and Methods) was used to immunoprecipitate MNDA and associated proteins from U937 and K562 cell nuclear protein extracts (Fig. 2). Immunoprecipitated proteins were visualized by immunoblot detection of MNDA (Fig. 2B) and nucleolin (Fig. 2C). Multiple staining bands migrating below the Mr 55,000 MNDA (Fig. 2B, arrow) are degradation products [Goldberger et al., 1986]. The K562 cell nuclear protein extract was devoid of MNDA (Fig. 2B, lane 2), while the initial K562 cell nuclear protein extract contained an amount of immunochemically active nucleolin similar to that in the U937 cells (Fig. 2C; compare lane 1 with lane 2). In the immunoprecipitation reactions, nucleolin was not recovered from the MNDA negative K562 cell extract (Fig. 2C, lane 4) but was recovered from the U937 cell extract (Fig. 2C, lane 3). The results demonstrate that natural MNDA interacts with nucleolin and MNDA is required for coimmunoprecipitation of nucleolin from nuclear protein extracts. The nuclear extracts from U937 and K562 cells contain multiple lower Mr bands of apparently degraded nucleolin (Fig. 2C, lanes 1 and 2). However, the coprecipitation reaction with MNDA recovered only the 100 kDa form of nucleolin (Fig. 2C, lane 3). This result indicates that the intact nucleolin is required for MNDA binding and therefore binding is not the result of nonspecific charge attraction.

Protein Blotting Analysis of the Specificity of rMNDA–100 kDa Protein Binding

A protein blotting/Far Western assay [Masson et al., 1993] was used to further assess the specificity of MNDA protein binding activity. Proteins in nuclear or cytosolic extracts, rMNDA, and affinity purified MNDA binding proteins (prepared using the rMNDA-Ab-bead complexes as in Fig. 1, lane 2) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membrane. The nonspecific protein binding sites were blocked [Masson et al., 1993] prior to incubation with [^{32}P]rMNDA. The rMNDA bound a 100 kDa band (Fig. 3, arrow) strongly in the nuclear protein extract of the MNDA-positive cell and bound the 100 kDa band to a lesser extent in the MNDA-negative cell extract (Fig. 3, lanes 4,6). The 100 kDa band that bound [^{32}P]rMNDA was also present in the affinity purified rMNDA binding protein preparation (Fig. 3, lane 5). This analysis also showed that rMNDA bound to itself (Fig. 3, lane 1, major band of activity) and to natural MNDA found in the U937 cell nuclear protein extract (Fig. 3, lane 4, arrowhead). The identification of the natural MNDA was confirmed by immunoblot analysis of the same sample (data not shown). These findings suggest that MNDA might function as a homodimer or multimer. In addition, MNDA bound to uniden-

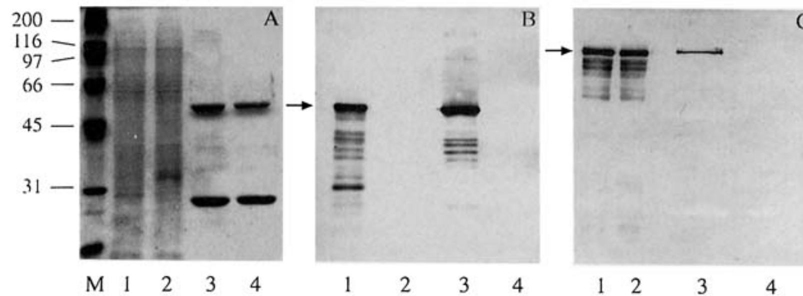

Fig. 2. SDS-polyacrylamide gel electrophoresis of MNDA and associated proteins immunoprecipitated from a U937 or K562 cell nuclear protein extracts with rat antibodies against rMNDA. **A:** *Lane M:* Protein markers with molecular weights indicated (kDa). Nuclear protein extract from U937 cells (*lane 1*), nuclear protein extract from K562 cells (*lane 2*), immunoprecipitated proteins from U937 (*lane 3*), and K562 cell nuclear protein extracts (*lane 4*). A 10% polyacrylamide gel was stained with Coomassie brilliant blue. **B:** Duplicate samples as in A, lanes 1–4, were reacted with anti-MNDA (rabbit polyclonal antiserum against rMNDA) in the immunoblot assay. **C:** Duplicate samples as in A, lanes 1–4, were reacted with rabbit polyclonal monospecific antibodies against nucleolin. The arrows designate MNDA in B and the 100 kDa nucleolin band in C. In C, the immunoprecipitated samples (*lanes 3, 4*) were analyzed at six times the equivalent amount of material as used in B (*lanes 3, 4*). These samples required adjustment to compensate for the less sensitive immunochemical detection of nucleolin relative to MNDA and the low abundance of MNDA compared to nucleolin. Rat heavy and light chains obtained in the immunoprecipitation (two major protein bands in lanes 3, 4 in A) were not recognized by immunoblot detection of MNDA or nucleolin (lanes 3, 4 in B, C).

tified proteins that appeared only in the nuclear protein extracts and to others that are present in both the cytosolic and nuclear protein fractions (Fig. 3; compare lane 2 with lane 4). The results of this assay showed that rMNDA bound directly to nucleolin, to other unidentified proteins, and to itself.

The effect of various treatments on the rMNDA protein binding activity was investigated (data not shown). Because MNDA is a basic protein with an isoelectric point of 9.5, some protein-protein binding might be expected to occur through electrostatic attraction to acidic proteins or to highly acidic regions within proteins as in the case of nucleolin [Olson, 1990]. However, no binding was evident in samples that were boiled (30 min) prior to electrophoresis or subjected to repeated freeze-thawing (more than twice). The rMNDA binding activity was resistant to 0.5 M NaCl and 1% nonionic detergent in the protein blotting assay even though these same conditions were effective in eluting the proteins bound to the rMNDA-Ab-bead complexes (Fig. 1, lane 2), indicating that the binding affinity is higher in the membrane-based assay. The coimmunoprecipitation reaction (Fig. 2C) also demonstrated that the MNDA binding was specific for the intact nucleolin and not partially degraded forms. These observations indicate that sequence and conformation rather than charge determines the MNDA protein binding activities observed in this study.

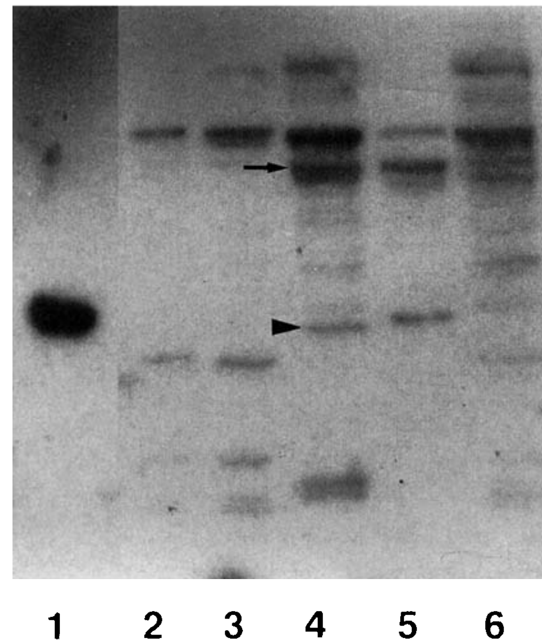

Fig. 3. A protein blotting analysis of [^{32}P]rMNDA binding. Recombinant MNDA (*lane 1*), U937 cytosolic protein fraction (*lane 2*), K562 cytosolic protein fraction (*lane 3*), U937 nuclear protein extract (*lane 4*), affinity purified MNDA binding proteins isolated from U937 cell nuclear protein extract (*lane 5*), and HeLa cell nuclear protein extract (*lane 6*). Binding of [^{32}P]rMNDA to the 100 kDa protein band (arrow, lane 4) was observed in nuclear protein extracts analyzed. Natural MNDA (arrowhead) is present in the U937 nuclear protein extract which also bound [^{32}P]rMNDA (lane 4).

DISCUSSION

Nucleolin (C23), a member of a family RNA-binding proteins including nucleophosmin/B23, is a ubiquitous, nucleolar protein thought to be involved in ribosome biogenesis [Olson, 1990]. Recent reports suggest that the function of nucleolin could be more diverse. Nucleolin has been identified as a nuclear target protein of the 25 kDa FK506 binding protein [Jin and Burakoff, 1993]. Nucleolin also represses expression of the liver *alpha-1 acid glycoprotein* (*AGP*) gene through sequence specific binding to the B element (5′-NTGYCCCNN-3′) [Yang et al., 1994]. The effect of nucleolin on the B element is further influenced by YY1, a human multifunctional factor that can act either positively or negatively on transcription depending on its interactions with other proteins [Momoeda et al., 1994]. YY1 was recently observed, in transient transfection assays, to activate *AGP* transcription by interfering with the repressor function of nucleolin bound to the B element [Lee and Lee, 1994]. The characterization of the phosphorylation of nucleolin on the cell surface by an ectoprotein kinase has suggested a role for nucleolin in communicating extracellular signals into the nucleus [Jordan et al., 1994]. Nucleolin also binds apo B– and apo E–containing lipoproteins in a manner similar to the LDL receptor [Semenkovich et al., 1990]. While most of the cellular nucleolin is concentrated in the nucleolus, it has been proposed that a subpopulation shuttles between the nucleus, cytoplasm, and cell surface providing a possible mechanism for extracellular regulation of nuclear events by lipids [Semenkovich et al., 1990]. The interaction between the FK506 binding protein and nucleolin [Rutherford and Zuker, 1994] as well as the recent demonstration of association with growth-associated factors [Take et al., 1994] further supports nucleolin's role in signaling [Rutherford and Zuker, 1994]. Nucleolin is also a specific nuclear matrix attachment region binding protein that is distributed between three nuclear protein fractions similar to other nuclear matrix proteins [Dickinson and Kohwi-Shigematsu, 1995]. These observations indicate that nucleolin functions outside of the nucleolus possibly in association with the nuclear matrix [Dickinson and Kohwi-Shigematsu, 1995].

The identification of motifs within MNDA responsible for binding nucleolin is an important goal. In this regard, a motif within a basic amphipathic α-helical region of MNDA [Briggs et al., 1992], amino acids 84–103, matches a proposed consensus LDL receptor binding sequence of apolipoprotein B-100 [Hospattankar et al., 1986]. A second similar motif is located near the C-terminus (amino acids 388–400) of MNDA. While these motifs might account for the specific interaction between MNDA and nucleolin and between nucleolin and lipoproteins, their presence also suggests a possible functional relationship between MNDA, nucleolin, and lipoprotein in myelomonocytic cell physiology. The importance of lipoprotein effects on monocyte function in atherosclerosis has long been recognized [Faruqi and DiCorleto, 1993]. Specific effects of lipoproteins on macrophage proliferation have also been recently documented [Sakai et al., 1994]. The involvement of MNDA in mediating such events could add cell specificity to the signaling pathway and/or to the cellular response.

At this time it is unclear how the specific MNDA protein-protein interactions relate to its possible role in myelomonocytic cell responses to interferon α. However, the involvement of nucleolin in an interferon signaling mechanism was proposed earlier [Williams, 1991]. In addition, relevance of the protein binding properties of MNDA compared to other members of its gene family remains unknown. It should be noted that the related human IFI 16 protein also contains a site (amino acids 699–712) matching the putative LDL receptor binding sequence found in apolipoprotein B-100 [Trapani et al., 1992].

The results of this study indicate that MNDA exhibits specific protein binding activities and the characterization of these interactions represents a new area for investigating function of MNDA and related gene products. The specific interaction between MNDA and nucleolin suggests a role for MNDA in intracellular signaling and/or nuclear structure.

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