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Identification and Characterization of a Leukocyte-specific Component of the Nuclear Body*

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The nuclear body (NB) is a cellular organelle that is involved in the pathogenesis of acute promyelocytic leukemia and viral infection. The NB is also a target of antibodies in the serum of patients with the autoimmune disease primary biliary cirrhosis. In this study, serum from a patient with primary biliary cirrhosis was used to identify a cDNA encoding a novel component of the NB, a 140-kDa protein designated Sp140. The predicted amino acid sequence of the amino-terminal portion of Sp140 was similar to Sp100, a previously identified NB protein. The carboxyl portion of Sp140 contained a zinc-finger domain and a bromodomain, motifs that are present in proteins regulating gene transcription. High levels of Sp140 mRNA were detected in human spleen and peripheral blood leukocytes, but not other human tissues. The level of Sp140 mRNA in myeloid precursor cell lines HL60 and NB4 markedly increased in response to chemically induced cellular differentiation. Immunohistochemical techniques were used to demonstrate that Sp140 localized to the NB in differentiated HL60 and NB4 cells. The location of Sp140 in the NB, and expression of this gene in cells involved in host defense, suggest that Sp140 may be involved in the pathogenesis of acute promyelocytic leukemia and viral infection.

The nuclear body (NB1; also known as nuclear domain 10, PML oncogenic domain, and Kr body) is a nuclear organelle whose function is unknown (1–5). Using immunohistochemical staining, NBs appear as 5–30 discrete, punctate, dotlike regions within the nucleus. The NB is distinct from other nuclear domains including those involved in DNA replication and mRNA processing. In addition, components of the NB do not co-localize with kinetochores or centromeres (6). The number of NBs in the cell and the intensity of antibody staining of these structures increase in response to stimuli including interferons (IFNs), heat shock, and viral infection (1).

The NB is a target of autoantibodies in the serum of patients with the autoimmune disease primary biliary cirrhosis (PBC). Approximately 40% of patients with PBC have antibodies directed against this structure (4, 5). Serum from patients with PBC was used to identify and characterize a 100-kDa component of the NB which was designated Sp100 (Speckled, 100-kDa) (6). Xie et al. (7) demonstrated that fusion of Sp100 to the LexA DNA binding domain activated gene transcription in Saccharomyces cerevisiae. The authors suggested that Sp100 may participate in activation of transcription of specific regions in the genome (7).

A second component of the NB, designated NDP52, was characterized using a murine monoclonal antibody that reacted with the NB (1, 8). A cDNA encoding NDP52 was identified, and the predicted amino acid sequence contained coiled-coil, leucine zipper, and zinc-finger motifs. One or more of these domains may be involved in interactions between NDP52 and other components of the NB (8).

A third component of the NB, designated PML, was identified by several investigators studying the t(15;17) translocation associated with acute promyelocytic leukemia (APL) (9–12). In this translocation, the amino-terminal portion of PML is fused to the retinoic acid receptor-α (RAR-α). PML was found to co-localize with Sp100 in the NB (13, 14). Expression of the PML-RAR-α fusion protein in APL cells appeared to disrupt the NB; in these cells, the NB antigens were detected in numerous smaller regions in the nucleus described as “microspeckles.” Treatment of APL cells with retinoic acid (RA) resulted in differentiation of myeloid precursor cells and reformation of NBs (3, 13, 14). In patients with APL, treatment with RA results in differentiation of leukemic cells and temporary disease remission (15).

PML, like Sp100 and NDP52, is expressed in a wide variety of human cell lines. Immunoreactive PML was detected in myeloid precursor cells as well as erythroblasts and megakaryocytes in the bone marrow (16). In addition, PML was detected in psoriatic skin lesions, hyperplastic breast and colon tissues, and in the endometrial mucosa during the follicular phase of the menstrual cycle (17). In recent studies, Sternsdorff et al. (18) demonstrated that PML, like Sp100, is a target of autoantibodies in patients with PBC.

Expression of the three previously identified components of the NB is enhanced by treatment with IFNs (8, 19, 20). Guldner et al. (19) suggested that enhanced expression of Sp100 by IFNs reflects a role for Sp100 in the “anti-viral state.” Doucas et al. (20) demonstrated that IFN-induced expression of PML in HEp2 cells...
Sp140 Component of the Nuclear Body

29199

was associated with inhibition of adenovirus replication.

The NB is a site of interaction between viral and host proteins. Maul et al. (21) and Maul and Everett (22) demonstrated that infection of cells with herpes simplex virus type I resulted in disruption of the NB. Using herpes simplex virus type I deletion mutants, disruption of the nuclear body was shown to be a result of the action of the viral protein ICP0. Recent studies have demonstrated that other viral proteins, including the adenovirus E4-ORF3 protein, also interact with this nuclear organelle (20, 23, 24).

In this study, serum from a patient with PBC was used to identify a cDNA encoding a third autoantigen in the NB. To keep with the nomenclature introduced for Sp100 and keeping with the nomenclature introduced for Sp100 and be-

MATERIALS AND METHODS

Human Serum—Serum was obtained from a patient with PBC. The diagnosis was based on the presence of elevated liver function enzymes and high titer antibodies directed against the mitochondrial antigen E2-pyruvate dehydrogenase complex (25).

Cell Culture and Induction of Differentiation—HL60 cells (American Type Tissue Collection, Rockville, MD) and NB4 cells (a gift of M. Lanotte, Institut National de la Sante et Recherche Medicale, Paris, France) were maintained in RPMI supplemented with 10% fetal calf serum, l-gluta-

Immunohistochemical Staining—Adherent cells were grown in tissue culture chambers (Nunc, Inc., Naperville, IL) and fixed in 4% paraformaldehyde in phosphate-buffered saline at room temperature for 15 min. Cytospin preparation of nonadherent cells was performed with 50,000 cells at 500 rpm for 5 min followed by air drying and fixation in paraformaldehyde. Prior to incubation with antibodies, cells were permeabilized with 0.1% saponin in phosphate-buffered saline and then treated with 0.6% hydrogen peroxide in 60% methanol to block endogenous peroxidase activity. Cells were immunostained with mouse monoclonal antibody directed against PML (a gift of K. van der Knaap, E. C. Slater Instituut, Amsterdam) (29) or rat antiserum directed against Sp140 using horseradish peroxidase-conjugated avidin-biotin complexes (ABC) (Vector ABC Elite Kit; Vector Laboratories). In some experiments, cells were stained with rat anti-Sp140 antibodies that were affinity-purified using recombinant protein (see below). Cells incu-

Isolation and Preliminary Analysis of cDNA Clones—Patient serum

diluted 1:200 in blocking solution (phosphate-buffered saline containing 5% nonfat dry milk, pH 7.4) was used to screen a λgt11 cDNA expres-

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formaldehyde-agarose gels (10 μg/lane) and transferred to nylon membranes. Membranes were hybridized with the radiolabeled SacII/EcoRI restriction fragment of the Sp140 cDNA. Membranes were washed and subjected to autoradiography. To confirm comparable loading of RNA in each lane, membranes were hybridized with a 10-fold molar excess of a 32P-labeled oligonucleotide complementary to rat 18 S RNA (39).

**RESULTS**

**Isolation of a cDNA Encoding Sp140—**Patients with the autoimmune disease PBC produce autoantibodies directed against proteins in the nuclear body. To identify novel components of this structure, serum from a patient with PBC was used to screen a λGT11 cDNA expression library prepared from HEPG2 cells. Approximately one million bacteriophages, one clone (Sp140.1) producing immunoreactive recombinant protein was identified. The nucleotide sequence of the cDNA was found to contain a 781-bp open reading frame (ORF) that extended for the entire length of the fragment, suggesting that the cDNA encoded only a portion of a protein. To identify a full-length cDNA, the DNA fragment was radiolabeled and used to rescreen the HEPG2 cDNA library; no additional clones were identified. The radiolabeled fragment was then used to screen a HUVEC cDNA library, and a single hybridizing cDNA (Sp140.2) was identified among one million bacteriophages. The clone contained a 2,732-bp insert that included the entire length of Sp140.1. A 2,193-nucleotide ORF extended to the 5’ end of Sp140.2, suggesting that this clone also encoded only part of the full-length protein. The 5’-RACE technique was used to identify the 5’ end of the mRNA encoding Sp140. The RACE product contained an additional 173-bp segment. The polymerase chain reaction product and the 5’ end of Sp140.2 were joined at a BstXI site, and the full-length cDNA was ligated into eukaryotic expression vector pCDNA3.

To confirm that the isolated cDNA encoded a protein that reacted with autoantibodies in the patient’s serum, the plasmid was used for in vitro transcription and translation of Sp140. Autoantibodies in the patient’s serum reacted with the 140-kDa translation product by immunoblot (Fig. 1, panel A, + lane). In contrast, this band was not detected in a lane containing reticulocyte lysate proteins alone (Fig. 1, panel A, – lane). These results demonstrated that pCDNA3-Sp140 encoded an immunoreactive protein.

**Sequence Analysis of Sp140—**The cDNA encoding Sp140 was 2,905 bp in length and contained an ORF from 107 to 2,365 (Fig. 2A). The start codon was preceded by an in-frame stop codon. Several features of the predicted amino acid sequence of Sp140 were of interest. The amino-terminal portion of Sp140, between amino acids 29 and 157, was 49% identical to the amino-terminal region of Sp100, a previously identified autoantigen within the NB (Fig. 2B). Several domains of Sp140 were positively charged. The central portion of Sp140, between amino acids 381–400, contained a potential bipartite nuclear localization sequence (40–42). The carboxyl portion of Sp140 contained a cysteine-rich, zinc-finger motif (amino acids 579–639). This region was followed by a segment (amino acids 681–715) that was similar to the bromodomain in S. cerevisiae protein BDF1 (Fig. 2C) (43–45). The presence of a strongly negatively charged region, zinc-finger motif and bromodomain suggested that Sp140 may be involved in the regulation of gene transcription.

**Expression of Sp140 in Human Tissues and Cell Lines—**The expression of the gene encoding Sp140 in human tissues was analyzed by RNA blot hybridization. High levels of Sp140 mRNA were detected in human spleen and peripheral blood leukocytes (Fig. 3). In contrast, much lower levels of Sp140 mRNA were observed in thymus, prostate, ovary, small intestine, and colon. Low levels of a slightly smaller transcript, possibly an alternatively spliced form of Sp140 mRNA, were detected in several tissues. In addition, a 1,200-nucleotide transcript of uncertain significance was detected in human testis. Very low levels of Sp140 mRNA were observed in human heart, brain, placenta, lung, liver, skeletal muscle, kidney, and pancreas (data not shown).

To investigate the expression of Sp140 in cells of the monocye/granulocyte lineage, RNA was prepared from the myeloid precursor cell lines HL60 and NB4 before and after induction of cellular differentiation. Treatment of HL60 cells with Me2SO induces a polymorphonuclear leukocyte morphology; treatment with PMA induces a monocyte-like morphology (26, 27). Low levels of Sp140 mRNA were detected in untreated HL60 cells (Fig. 4, lanes 1 and 4). The level of mRNA was increased by treatment of HL60 cells with either PMA (lane 2) or Me2SO (lane 5). Treatment of human APL cell line NB4 with all-trans retinoic acid (RA) is also associated with cellular differentiation (28). Low levels of Sp140 mRNA were detected in NB4 cells prior to treatment with RA (Fig. 4, lane 7). In contrast, 5 days after addition of RA (1 μM), the level of Sp140 mRNA was markedly increased (lane 8). These results demonstrated that differentiation of HL60 and NB4 cells was associated with increased expression of Sp140.

**IFN Treatment Induces Expression of Sp140—**The expression of previously characterized components of the NB was enhanced by treatment of cells with IFN (8, 17, 19, 20). To investigate the effect of IFN on the level of Sp140 mRNA, HL60 cells were treated with IFN-γ (200 units/ml). Increased Sp140 mRNA levels were detected 24 h after treatment and persisted for at least 4 days (Fig. 5). In addition, treatment with IFN-γ further increased Sp140 mRNA in cells treated with PMA (Fig. 4, lane 3) or Me2SO (Fig. 4, lane 6). These results demonstrated that, as with Sp100, NDP52, and FML, expression of Sp140 was enhanced by IFN-γ.

**Cellular Localization of Sp140—**To permit cellular localization of Sp140 by immunohistochemical techniques, antiserum directed against a recombinant fragment of the protein was prepared. Three rats were immunized with Sp140 amino acids 131–391 fused to GST (GST-Sp140-(131–391)). The resulting antisera reacted with the in vitro translation product of
Fig. 2. Panel A, the nucleotide and predicted amino acid sequences of Sp140. The region of similarity between the amino-terminal portions of Sp140 and Sp100 is indicated in italics. A presumed nuclear localization sequence is present between amino acids 381–400 (indicated by a box). Conserved cysteine/histidine residues in the zinc-finger motif are circled. The bromodomain between amino acids 681 and 715 is underlined. These sequencedata are available from GenBank™/EMBL/DDBJ under accession no. U63420.

Panel B, comparison between the amino-terminal regions of Sp140 and Sp100. Forty-nine percent of the amino acids in this region are identical.

Panel C, comparison between Sp140 amino acids 681 and 715 and a bromodomain in S. cerevisiae protein BDF1. Seventeen of 35 amino acids in this region are identical.
In addition, the antisera reacted with a 140-kDa band in an immunoblot prepared from Me2SO-treated HL60 cells (Fig. 6, lane 1). Expression of Sp140 mRNA was enhanced 1, 2, and 4 days after addition of IFN-γ (lanes 2, 3, and 4). To confirm comparable loading of RNA in each lane, membranes were hybridized with a 10-fold molar excess of 32P-radiolabeled oligonucleotide complimentary to 18 S RNA.

This is the same pattern as that produced by mouse monoclonal antibodies directed against PML (panel B). Pretreatment of rat anti-Sp140 antibodies with recombinant protein completely blocked the NB staining pattern (panel C). Two-color immunohistochemistry demonstrated that rat anti-Sp140 antibodies and mouse anti-PML antibodies localized to the same structures in individual cells (panel D).

To investigate the cellular distribution of Sp140 in APL cell line NB4, rat antisera was used to stain NB4 cells before and after treatment with RA. Rat antisera directed against Sp140 stained NBs in NB4 cells that were treated for 5 days with RA (Fig. 7, panel E). Mouse monoclonal antibody directed against PML produced the same pattern of staining in RA-treated NB4 cells (panel F). Rat anti-Sp140 antisera did not stain the nuclei of untreated NB4 cells (panel G).

DISCUSSION

Patients with the autoimmune disease primary biliary cirrhosis produce antibodies directed against components of a cellular organelle known as the nuclear body. Two proteins in the NB, Sp100 and PML, have previously been shown to be targets of autoantibodies. In this study, serum from a patient with PBC was used to identify a novel NB antigen which was designated Sp140. The amino-terminal portion of Sp140 was 49% identical to the amino-terminal region of Sp100. These portions of Sp100 and Sp140 contain potential coiled-coil motifs (data not shown). Koken et al. (14) hypothesized that the amino-terminal domain of
Sp100 was a likely region for interactions between Sp100 and “other PBC autoantigens.” A direct interaction between Sp100 and Sp140 has not yet been demonstrated.

The region in Sp140 between amino acids 579 and 619 contained a cysteine-rich, zinc-finger motif. The presence of a zinc-finger domain in Sp140 suggests that the protein is a member of a larger family of proteins that have been implicated in the control of development, cellular differentiation, and cell growth. The zinc-finger region may bind DNA directly or may interact with a second protein to form a dimer that binds DNA (46–48). The order of the cysteine/histidine residues in Sp140 (C4HC3) differed slightly from that of the previously described RING finger (C3HC4) and LIM motif (C2HC5). Koken et al. observed that the C4HC3 motif is present in a variety of proteins including Drosophila trithorax and its human homologue ALL-1 and proposed the name TTC (for trithorax consensus) for this family of proteins (49).

The portion of Sp140 between amino acids 681 and 715 contained a bromodomain. The bromodomain is a conserved sequence that is found in a variety of transcriptional regulatory proteins (43–45). The predicted secondary structure of the bromodomain includes two strongly amphipathic $\alpha$ helices followed by reverse turns. Although the functional significance of the bromodomain is unknown, the hydrophobic surfaces of the helices may serve as sites of intramolecular protein-protein interaction. These interactions may influence the assembly or activity of multicomponent complexes involved in transcriptional activation (50).

Expression of Sp140 in Human Tissues and Cell Lines—High levels of mRNA encoding Sp140 were detected in human spleen and peripheral blood leukocytes and much lower levels were detected in all other tissues examined. The predominant expression of Sp140 in human leukocytes suggests that Sp140 may have an important role in cellular functions that are unique to these cells. Low levels of Sp140 mRNA were detected in the myeloid precursor cell lines HL60 and NB4. Expression of Sp140 in HL60 cells was markedly increased during the course of differentiation of these cells toward either monocytes or polymorphonuclear leukocytes. In addition, expression of Sp140 in NB4 cells was markedly increased following treat-
that Sp100 may be involved in establishing an "anti-viral
mechanism of action of RA in these cells is unknown. The myeloid
NB and induces leukemic cell maturation (3,9–14). The mech-
anism and translation. In addition, the antiserum reacted in
immunoblot with Sp140 in Me2SO-treated HL60 cells. By im-
munohistochemical staining techniques, Sp140 co-localized
with PML in Me2SO-treated HL60 cells. These results dem-
strate that Sp140 is a component of the NB.

Sp140 and Acute Promyelocytic Leukemia—In APL, a trans-
location between chromosomes 15 and 17 results in fusion of the
NB protein PML to RAR-α. Expression of the fusion protein disrupts
the NB and inhibits normal myeloid maturation. Treatment of APL cells with RA results in reformation of the NB and induces leukemic cell maturation (3, 9–14). The mech-
anism of action of RA in these cells is unknown. The myeloid
precursor cell line NB4 contains the t(15;17) translocation asso-
ciated with APL (28). Using immunohistochemistry, Sp140 was not detected in untreated NB4 cells (Fig. 7G). This was not surprising in view of the low level of Sp140 mRNA in these cells (Fig. 4). However, following treatment of NB4 cells with RA, Sp140 was detected within the NBs of these cells (Fig. 7, panel E). The observations that expression of Sp140 in NB4 cells is induced by RA treatment and that Sp140 associates with the
NB suggest that Sp140 may have a role in the action of RA on
APL cells. The observed resistance of APL cells to RA that
is expressed in some, but not all, lymphocyte cell lines is not known.

In summary, a novel component of the NB has been identi-
ified and characterized. The predicted amino acid sequence of Sp140 suggests that this protein is involved in the regulation of gene expression. The predominant expression of Sp140 in leukocytes raises the possibility that this protein can be regulated in cellular functions that are unique to these cells. Future studies of Sp140 may provide insight into the function of the NB and the role of this organelle in myeloproliferative disease, viral infection and autoimmune disorders.

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