

Hypoxia-activated ligand HAL-1/13 is lupus autoantigen Ku80 and mediates lymphoid cell adhesion in vitro

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Received 10 February 2000; accepted in final form 6 November 2000

Lynch, Eileen M., Robert B. Moreland, Irene Ginis, Susan P. Perrine, and Douglas V. Faller. Hypoxia-activated ligand HAL-1/13 is lupus autoantigen Ku80 and mediates lymphoid cell adhesion in vitro. *Am J Physiol Cell Physiol* 280: C897–C911, 2001.—Hypoxia is known to induce extravasation of lymphocytes and leukocytes during ischemic injury and increase the metastatic potential of malignant lymphoid cells. We have recently identified a new adhesion molecule, hypoxia-activated ligand-1/13 (HAL-1/13), that mediates the hypoxia-induced increases in lymphocyte and neutrophil adhesion to endothelium and hypoxia-mediated invasion of endothelial cell monolayers by tumor cells. In this report, we used expression cloning to identify this molecule as the lupus antigen and DNA-dependent protein kinase-associated nuclear protein, Ku80. The HAL-1/13-Ku80 antigen is present on the surface of leukemic and solid tumor cell lines, including T and B lymphomas, myeloid leukemias, neuroblastoma, rhabdomyosarcoma, and breast carcinoma cells. Transfection and ectopic expression of HAL-1/13-Ku80 on (murine) NIH/3T3 fibroblasts confers the ability of these normally nonadhesive cells to bind to a variety of human lymphoid cell lines. This adhesion can be specifically blocked by HAL-1/13 or Ku80-neutralizing antibodies. Loss of expression variants of these transfectants simultaneously lost their adhesive properties toward human lymphoid cells. Hypoxic exposure of tumor cell lines resulted in upregulation of HAL-1/13-Ku80 expression at the cell surface, mediated by redistribution of the antigen from the nucleus. These studies indicate that the HAL-1/13-Ku80 molecule may mediate, in part, the hypoxia-induced adhesion of lymphocytes, leukocytes, and tumor cells.

lupus antigen; lymphocyte; fibroblast

ENDOTHELIAL CELLS FROM DIFFERENT SOURCES increase their adhesiveness to leukocytes when subjected to hypoxia (3, 20, 46, 51, 58, 69, 73). We have also shown that human muscle rhabdomyosarcoma (RD) cells respond to hypoxia in the same way, becoming more adhesive for leukocytes (19). In a functional screen of antibodies generated against membrane antigens on hypoxic human endothelial and RD cells, we identified and characterized a new cell surface molecule, hypoxia-activated ligand (HAL-1/13), which is expressed on both endothelial and RD cells and mediates the

increased adhesion of leukocytes to both RD cells and endothelium under hypoxic conditions (21).

In addition, HAL-1/13 was shown to be expressed on the surface of several leukemia or lymphoma cell lines, including Jurkat T cell leukemia and U-937 histiocytic lymphoma, as well as on the surface of solid tumor cells, including hepatoma (Hep 3B) cells and several neuroblastoma and breast carcinoma cell lines (18). We also recently reported that hypoxic exposure of Kelly neuroblastoma and MCF7 breast carcinoma cells resulted in upregulation of HAL-1/13 surface expression, coincident with an increased ability of these tumor cells to invade endothelial monolayers. This enhanced invasion could be partially attenuated by the anti-HAL-1/13 antibody. Anti-HAL-1/13 antibody also inhibited locomotion of hypoxic tumor cells on laminin (18). These studies indicate that the HAL-1/13 antigen plays a crucial role in cell-cell and perhaps also cell-matrix interactions under hypoxic conditions.

To elucidate the nature of the HAL-1/13 antigen and mechanism of its surface upregulation upon hypoxic treatment, the antigen recognized by the HAL-1/13 monoclonal antibody (MAb) was isolated by expression cloning. We report here that the sequence of the HAL-1/13 antigen is identical to the p80/p86 subunit (Ku80) of the DNA-binding protein, Ku, previously identified as a target for autoantibodies produced by patients with systemic lupus erythematosus and related rheumatic disorders (41, 42, 52, 53). Ku80 has also been identified as a regulatory subunit of the DNA-dependent protein kinase (10, 15, 22, 25). In addition, antibodies raised against Ku80 recognize the HAL-1/13 antigen. Furthermore, we demonstrate that transfection of HAL-1/13-Ku80 into (murine) NIH/3T3 fibroblasts results in its expression on the plasma membrane, with concomitant induction of adhesive properties in the transfectants for human lymphoid cells, in a HAL-1/13-Ku80-dependent fashion. These data conclusively demonstrate the ability of HAL-1/13-Ku80 to function as an adhesion molecule. We also present evidence that hypoxic conditions upregulate cell surface expression of HAL-1/13-Ku80 via intracellular redistribution of HAL-1/13-Ku80 to the plasma membrane.

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MATERIALS AND METHODS

Molecular cloning and expression plasmids. The *Escherichia coli* strain XL1 Blue (Stratagene, La Jolla, CA) was used for all λ -phage work, and DH5 α (Life Sciences, Gaithersburg, MD) was used in the transfection and preparation of plasmid DNAs. A λ -ZAP (Stratagene) cDNA library of MCF7 cells [complexity of 1×10^7 ; generous gift of Dr. Mark Sobel, National Cancer Institute (9)] was screened with expression cloning after isopropyl β -D-thiogalactopyranoside (IPTG) induction (40) with the MAb HAL-1/13 (21). Recombinant phage were plated at a density of 50,000 plaque-forming units per 150-mm petri dish. Plaque lifts were prepared on 135-mm nitrocellulose discs (Schleider and Scheull, Keene, NH) as described (40). The membrane filters were subjected to Western blots, and positive plaques were isolated and purified to homogeneity (40). Plasmids were prepared from candidate bacteriophage clones by cotransfection of XL1 Blue with the helper M13 phage R407K and the λ -phage. The resulting phagemids were transfected into DH5 α , positive clones were selected and screened, and plasmid preparations were prepared. The insert DNA was sequenced in the Boston University DNA Core Facility with an Applied Biosystems International 3000 DNA sequencer.

A 2.79-kb *EcoRI/StuI* DNA fragment of pPRNZ-19 containing 0.04 kb of 5'-untranslated sequence, the entire reading frame of human Ku80 (2.16 kb), and 0.54 kb of 3'-untranslated sequence were subcloned into *EcoRI/SmaI*-digested pCINeo (Promega, Madison, WI). The resulting pCINeo-PRNZ vector contained untranslated human β -globin sequences upstream of the Ku80 ATG (40 bp 5' of the *EcoRI* site) that included an intron. Expression plasmids derived from pCINeo have been shown to produce increased amounts of RNA that enhance expression upon transfection. To generate Ku80 antisense constructs, a 2.39-kb *XhoI/EcoV* DNA fragment of pPRNZ-19 encompassing 1.54 kb of the open reading frame of human Ku80 and 0.85 kb of 3'-untranslated region were subcloned into *XhoI/SmaI*-digested pCINeo.

Cell lines and culture conditions. HeLa, a human cervical carcinoma cell line, U-937, a line derived from a histiocytic lymphoma with myelomonocytoid characteristics (62), and MCF7 (HTB 22), a human breast carcinoma cell line, were purchased from American Type Culture Collection (ATCC; Rockville, MD) and grown in Dulbecco's modified Eagle's medium (DMEM). DMEM was supplemented with 10% fetal bovine serum (FBS; Sigma Chemical, St. Louis, MO). NIH/3T3, a murine fibroblast cell line, was grown in DMEM supplemented with 10% donor calf serum (DCS). Jurkat, a T cell leukemia line (68), was grown in DMEM with 10% newborn calf serum (NCS). JY, a human Epstein-Barr virus (EBV)-transformed B cell line (39), was grown in RPMI 1640 supplemented with 10% FBS. All DMEM and RPMI 1640 were further supplemented with L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μ g/ml; Life Technologies, Grand Island, NY) and buffered to pH 7.4 with sodium bicarbonate. Cells were grown at 37°C in a humidified 5% CO₂ atmosphere and were in a log phase of cell growth when used in adhesion assays. All nonadherent cell lines were resuspended at 2×10^6 cells/ml, labeled with 2 μ M 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF; Molecular Probes, Eugene, OR) for 20 min at 37°C, washed, and resuspended in normoxic DMEM/NCS. RD cells, a human muscle cell line (38), were obtained from ATCC and grown using the same medium. MCF, 3T3, and RD cell lines were grown to confluence on 10-cm cell culture dishes, trypsinized, washed, and plated onto 96-well flat-bottom microtiter plates

(Nunc, Nunc, Denmark) at $\sim 1-2 \times 10^5$ cells/well and used 24-48 h later (when confluent) in adhesion experiments.

Flow cytometry and antibodies. The HAL-1/13 murine MAb (IgG2a) has been previously described (17). W6/32 is an IgG2a directed against a human leukocyte antigen class I framework antigen. Anti-Ku80 (MAbs 111, N9C1, and S10B1), anti-Ku70 (MAb H3H10), and anti-Ku70/80 (MAb 162) murine MAbs were manufactured by Neomarkers (Fremont, CA) and purchased from Lab Vision. Secondary antibody for fluorescence-activated cell sorter (FACS) analysis cell surface staining was a fluorescein-conjugated sheep (Fab')₂ fragment to mouse IgG (whole molecule; Cappel).

Flow cytometry of fluorescently labeled cells was performed on a Becton Dickinson FACScan (San Jose, CA), as previously described (17, 19, 20). Briefly, cells (1×10^6) were washed twice with PBS, collected, resuspended in Earle's balanced salts (EBSS)/0.5% serum, and pelleted gently, and the pellet was resuspended in 50-100 μ l of primary antibody diluted as required [no dilution for hybridoma supernatant (HAL-1/13 and W6/32); 1:100 dilution for ascites fluid (anti-Ku80, anti-Ku70, and anti-Ku70/80)]. The suspension was incubated with gentle mixing for 45 min at 4°C and then washed three times with 100 μ l of ice-cold EBSS/0.5% serum to remove excess primary antibody. The pellet was resuspended in 50-100 μ l of diluted secondary antibody, fluorescein isothiocyanate-conjugated sheep (Fab')₂ fragment to mouse IgG (whole molecule) at a 1:25 dilution, incubated with gentle mixing for 45 min at 4°C, washed three times with wash buffer, and resuspended in 200 μ l of wash buffer to which 200 μ l of 2% paraformaldehyde was added to fix the cells. Samples were stored covered at 4°C before FACS analysis. A W6/32-stained sample was included in each experiment as a positive control for staining.

Hypoxic treatment of cells. Subconfluent cell cultures were covered with fresh culture medium (DMEM/10% FBS) that contained 20 mM HEPES to prevent a pH drop during hypoxic exposure. They were incubated in chambers that contained gas mixtures of 5% CO₂-95% N₂-0% O₂ (BOC Gases, Boston, MA) for various intervals at 37°C (attaining a measured Po₂ = 10-30 Torr in the medium). Chambers were regassed for 1 h after 24 h, when cells were required for 48-h experiments. Cell viability was maintained under these hypoxic conditions. Incubation of tumor cells under these hypoxic conditions had no significant effect on their viability: 90% of cells were alive at the end of incubation, as measured by trypan blue exclusion. Cells continued to proliferate after reoxygenation.

Transfections. To generate cell lines stably expressing HAL-1/13-Ku80, 3T3 cells were cotransfected with 30 μ g of the pCINeo-PRNZ vector (or the empty pCINeo vector as a control). Twenty-four hours before transfection, 3T3 cells were plated in 60-mm tissue culture plates at a density of 2.5×10^5 cells/plate. Cells were transfected with PRNZ DNA using Lipofectamine Plus (GIBCO Life Technologies). DNA (5 μ g), PLUS reagent (10 μ l), and serum-free medium (235 μ l) were mixed together and incubated for 15 min at room temperature. Lipofectamine (15 μ l) and serum-free medium (235 μ l) were mixed and added to the DNA-containing mixture. The Lipofectamine/DNA mixture was vortexed briefly and incubated for 15 min at room temperature. Growth medium was removed from the 60-mm tissue culture plates and replaced by 2 ml of serum-free medium. The Lipofectamine/DNA mixture was added to the cells and incubated for 3 h at 37°C. Serum-free medium was replaced with complete growth medium, and cells were selected 24-48 h later using DMEM/10% DCS supplemented with geneticin (G418; GIBCO Life Technologies) at 0.5 mg/ml. Medium was

changed regularly to remove dead cells. Colonies were selected for cloning 10–14 days after transfection. G418-resistant colonies were screened for cell surface expression of HAL-1/13-Ku80 by FACS analysis. Transfected cells were maintained in medium containing G418 at 0.5 mg/ml. Experiments were performed using pooled colonies of G418-resistant cells (to avoid artifacts due to clonal selection of aberrant cells) or clones derived from G418-resistant colonies.

Adhesion assay. Cell adhesion assay was a modified method of an adhesion centrifugation assay previously described (19, 20). Monolayer cells ($1-2 \times 10^5$ cells/ml) were plated in flat-bottomed 96-well tissue culture plates (Nunc) and exposed to either normoxic or hypoxic conditions for 24 or 48 h before cell adhesion assay. Normoxic and hypoxic tumor cells were washed twice with ice-cold EBSS/0.5% BSA and centrifuged at 1,000 rpm for 5 min at 4°C. The cell pellet was resuspended at 5×10^6 cells/ml in PBS/0.5% BSA, and calcein (BCECF-AM) was added to 2 μ M. Calcein-labeled cells were incubated for 25 min at 37°C, washed with PBS/0.5% BSA three times, and added to cell monolayers in 96-well plates (100 μ l/well). Plates were incubated for 30 min at 37°C. Adhesion of calcein-labeled cells to monolayer cells was visualized by both light and fluorescence microscopy before discarding the residual nonadherent cells and washing the plate twice with PBS/0.5% BSA. Quantitation of adherent cells was performed by measuring the fluorescence in each well with a CytoFluor 2300 plate scanner (Millipore, Burlington, MA) at excitation and emission wavelengths of 485 and 530 nm, respectively. Background fluorescence was measured for each plate and subtracted from all readings. Because the intrinsic labeling efficiency by calcein differs among cell types, adhesion data are expressed in “relative adhesion units” rather than absolute values to facilitate comparison of adhesion experiments among cell types. Adhesion (baseline fluorescence) of each cell line to the parental NIH/3T3 cells was arbitrarily given a value of one adhesion unit. Changes in adhesion (fluorescence) values under different experimental conditions are expressed relative to this baseline value of one. In blocking experiments using MAbs, anti-HAL-1/13 (diluted 1:1) or anti-Ku80 (diluted 1:25) MAbs were incubated with cell monolayers for 30 min at 37°C. The monolayers were washed twice, calcein-labeled cells were added, and cell adhesion was assayed. Each experiment was performed in triplicate and the SD calculated.

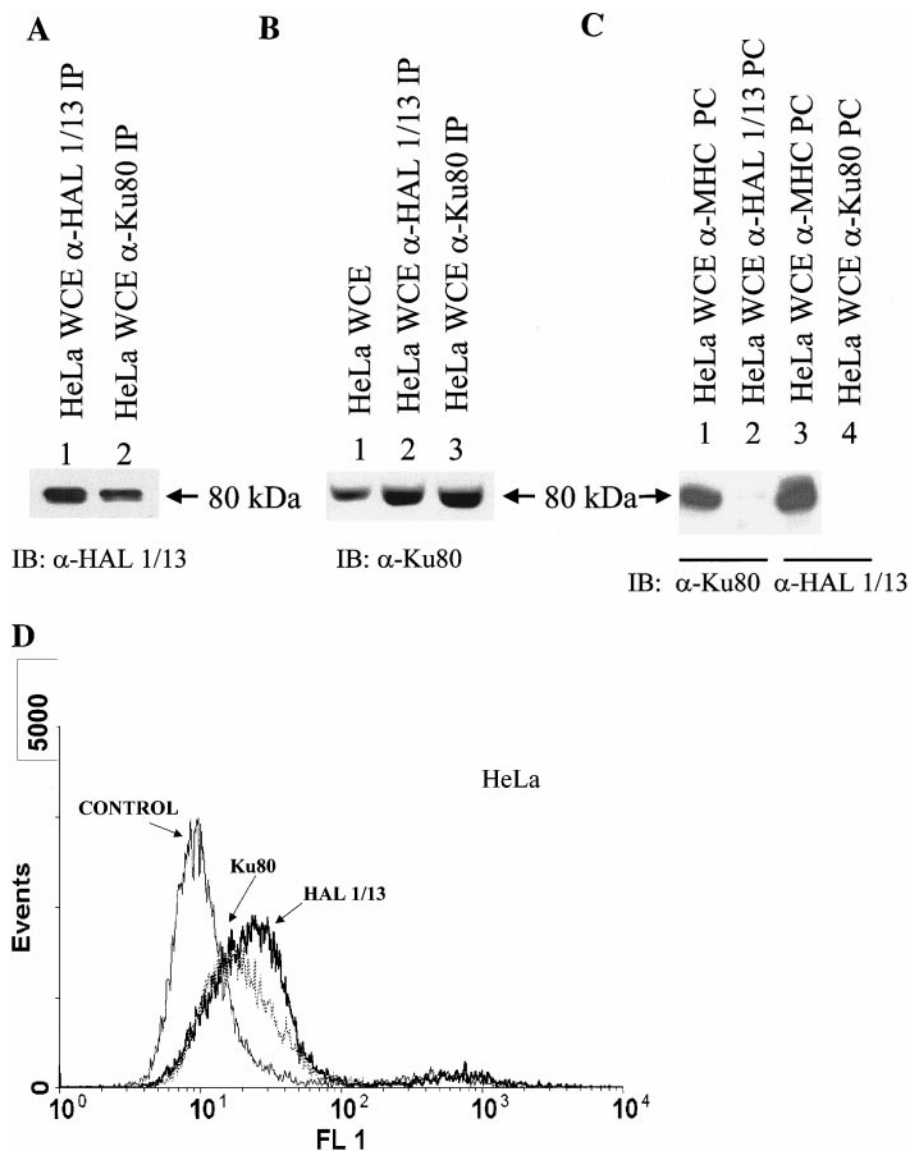
Preparation of membrane, nuclear, cytoplasmic, and whole cell extract fractions. The membrane, cytoplasmic, nuclear, and whole cell extract fractions were prepared separately for immunoprecipitation studies. After collection of $3 \times 10^7-4.5 \times 10^7$ cells, cells were washed and the cell pellet was resuspended in 10 ml of ice-cold PBS, vortexed briefly, and spun down. A minimal volume (0.5 ml/ 1×10^7 cells) of ice-cold lysis buffer [10 mM Tris (pH 7.5), 1% Triton X-100, 5 mM EDTA, 50 mM NaCl, 10 μ g aprotinin/ml, 10 μ g leupeptin/ml, and 100 μ M phenylmethylsulfonyl fluoride (PMSF)] was added to the cell pellet, which was then vortexed for 1 h at 4°C and centrifuged at 2,500 rpm for 10 min to pellet nuclei and debris. The supernatant (cytoplasmic fraction) was removed to a fresh microcentrifuge tube and centrifuged at 11,000 rpm for 30 min to remove debris, and the supernatant was stored at -80°C . The pellet (nuclear fraction) was resuspended in 0.5–1 ml of nuclear lysis buffer [10 mM Tris (pH 7.5), 1% Triton X-100, 5 mM EDTA, 500 mM NaCl, 10 μ g aprotinin/ml, 10 μ g leupeptin/ml, and 100 μ M PMSF] and vortexed for 15 min at 4°C. The supernatant was removed

and stored at -80°C . The whole cell extract fractions were prepared by combining separately prepared nuclear and cytoplasmic fractions in a ratio proportional to their extraction volumes (1:3). Protein concentration was determined using a Bio-Rad assay standardized against a BSA curve. For preparation of membrane fractions, subconfluent cells were washed and pelleted, and the cell pellet was resuspended in 1.0 ml of TMSDE [50 mM Tris (pH 7.6), 75 mM sucrose, 6 mM MgCl_2 , 1 mM dithiothreitol, 1 mM EDTA, 10 μ g aprotinin/ml, and 10 μ g leupeptin/ml] and incubated at 0°C for 10 min. Samples were frozen at -70°C , freeze-thawed at least three times, and then sheared using a 27-gauge needle and tuberculin syringe (8–10 times on ice). Nuclei and debris were removed by centrifugation at 13,000 rpm for 20–30 min at 4°C. The supernatant (membrane fraction) was spun at 45,000 g (22,000 rpm) for 1 h at 4°C. The pellet was resuspended in 0.1–0.2 ml TMSDE. Protein concentration was quantitated using a Bio-Rad assay standardized against a BSA curve. Membrane fractions were stored at -70°C in 10- to 20- μ l aliquots.

Immunoprecipitation and immunoblotting. Protein G-Sepharose beads (Sigma) were resuspended in lysis buffer (62.5 mg/ml) and washed three times before use. The lysates (and mock lysates: PBS/10% serum) were precleared by adding 50 μ l of protein G-Sepharose/ml of lysate in polypropylene tubes. The mixture was shaken with an orbital shaker either for at least 1 h or overnight at 4°C and then centrifuged at 1,565 rpm (200 g) for 5 min at 4°C to remove the beads. The supernatant (precleared fraction) was removed to a fresh microcentrifuge tube to which primary antibody was added. The primary antibody was added to the precleared lysate and mixed for 2 h at 4°C. Anti-HAL-1/13 mouse monoclonal hybridoma culture supernatant was used in a 1:1 ratio of MAb to lysate. For mouse ascites MAbs anti-Ku80, anti-Ku70, or anti-Ku70/80, 3–5 μ l of MAb were added per milligram of protein lysate. Protein G-Sepharose (62.5 mg/ml) was added to the lysate-antibody mixture (50 μ l/ml) and mixed for 1 h at 4°C. The mixture was centrifuged at 14,000 rpm for 5 min to precipitate the antigen. The immunoprecipitated (IP) fraction was washed two to three times with immunoprecipitation buffer [50 mM Tris-Cl (pH 7.5), 150 mM NaCl, 0.02% (wt/vol) sodium azide, 100 μ g PMSF/ml, 1 μ g aprotinin/ml (0.5%), 0.1% Tween 20, and 1 mM EDTA (pH 8.0)]; 1 ml buffer was added and centrifuged at 14,000 rpm for 2 min. The supernatant (immunodepleted fraction) and IP fractions were stored at -80°C .

Electrophoretic separation of proteins and transfer to nitrocellulose filters was performed as previously described (66). The nitrocellulose membrane was Ponceau stained and destained to assess equal protein loading. Blots were blocked with 5% nonfat milk, 10 mM Tris (pH 7.4), 100 mM NaCl, and 0.1% Tween 20 for 2 h at 4°C. The primary antibody (anti-HAL-1/13 mouse MAb; diluted 1:5), anti-Ku80, or anti-Ku70 MAb (diluted 1:1,000) was prepared in blocking solution and incubated with membrane for 1 h at room temperature. The membrane was washed with blocking buffer three to four times. The secondary antibody was horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H + L; Immunopure; Pierce) diluted 1:10,000 in blocking buffer and incubated for 45 min at room temperature. The membrane was washed with buffer three to four times and then air-dried briefly on blotting paper. The reaction was detected using an enhanced chemiluminescence (ECL) kit (Amersham) and autoradiography. Secondary antibody for ECL molecular weight markers used was streptavidin HRP NEL 750 (diluted 1:3,000). Band intensities in autoradiograms were quantitated by laser densitometry.

Fig. 1. Reciprocal antigenic cross-reactivity of the molecule identified by the hypoxia-activated ligand-1/13 (HAL-1/13) monoclonal antibody (MAb) by an anti-Ku80 MAb. One milligram of total cellular protein (whole cell extracts, WCE) from HeLa cells was left untreated (HeLa WCE) or was immunoprecipitated (IP) with the HAL-1/13 MAb (α -HAL-1/13 IP) or with a Ku80 MAb (Mab 111; α -Ku80 IP). Twenty-microgram aliquots of proteins were immunoprecipitated, and the complexes were separated by 7.5% SDS-PAGE, transferred, and immunoblotted (IB) with the HAL-1/13 MAb (hybridoma supernatant diluted 1:5; A) or with a Ku80 MAb (ascites diluted 1:1,000; B). In C, 20- μ g aliquots of whole cell lysates were first pre-cleared (PC) with an isotype-matched (IgG2a) control antibody (W6/32), recognizing a framework epitope on human class I myosin heavy chain (MHC) (lanes 1 and 3), the HAL-1/13 antibody (lane 2), or the anti-Ku80 antibody (lane 4) plus protein G-Sepharose. The PC lysates were then separated, transferred, and immunoblotted with the α -Ku80 MAb (lanes 1 and 2) or the HAL-1/13 MAb (lanes 3 and 4), as described above. The blots were developed with a horseradish peroxidase-conjugated secondary antibody (diluted 1:1,000) and an enhanced chemiluminescence (ECL) reagent. The autoradiographs produced are shown here. The migration position of an 80-kDa molecular mass marker is shown. D: cell surface expression of the HAL-1/13 and Ku80 antigens. Unfixed HeLa cells were stained with the undiluted HAL-1/13 MAb hybridoma supernatant (HAL-1/13), with an α -Ku80 MAb (Mab 111) diluted 1:100 (Ku80), or with no primary antibody (control), and then with an FITC-conjugated sheep (Fab')₂ anti-mouse IgG. Flow cytometric profiles are shown.



To assess the purity of the subcellular fractions, cross-contamination was quantitated by the immunoblotting of 20 mg of protein from each of the individual fractions of U-937 cells after electrophoretic separation of proteins and transfer to nitrocellulose filters. Antibodies against histone H3, glyceraldehyde-6-phosphate dehydrogenase, and insulin receptor were used to identify markers specific for nuclear, cytoplasmic, and membrane compartments, respectively. On a microgram per microgram of protein basis, nuclear contamination of membrane fractions averaged 12%, and membrane contamination of nuclear fractions was <5%. (Cytoplasmic fractions were not used in these studies because little or no HAL-1/13-Ku80 or Ku70 was detectable in the cytoplasm. Cytoplasmic contamination of nuclear fractions was 16%; cytoplasmic contamination of membrane fractions was 22%.)

Reproducibility and statistical analysis. All immunoblots and FACS histograms shown are representative of results from experiments that were performed at least three times. Similar results were obtained on each occasion. All data points shown are means of duplicates or triplicates, which differed by <5% unless stated otherwise.

RESULTS

Cloning of gene product recognized by HAL-1/13 antibody and identification of the antigen as human Ku80. Expression cloning was used to isolate the cDNA encoding the HAL-1/13 antigen. A λ -ZAP cDNA library of MCF7 cells (complexity of 1×10^7 genes) was screened using expression cloning after IPTG induction. Plaque lifts on membranes were probed with the MAb HAL-1/13, and positive plaques were isolated and purified to homogeneity. Candidate phagemids were rescued, and plasmid preparations were prepared and sequenced. One clone, pPRNZ-19, had a 3.05-kb *EcoRI/XhoI* insert that, by BLAST searches, was identical to human Ku80 (72). Expression of this clone in *E. coli* yielded a protein of \sim 80 kDa, which was immunoreactive with the HAL-1/13 MAb. This clone was used for all subsequent manipulations. Other shorter clones were also obtained, which were immunoreactive with

HAL-1/13 MAb, had nucleotide sequence identical to human Ku80 cDNA, and which may represent a truncated Ku80 transcript previously identified (6, 30).

To confirm that the HAL-1/13 antibody recognizes the Ku80 antigen, we performed reciprocal immunoprecipitations and immunoblots using HeLa cell extracts in which Ku antigen has been extensively studied (7). Both the HAL-1/13 antibody and the Ku80 antibody precipitated an 80-kDa protein from HeLa cell extracts that was recognized by the HAL-1/13 antibody (Fig. 1A). In reciprocal experiments, the Ku80 antibody recognized an 80-kDa protein immunoprecipitated by both the HAL-1/13 antibody and the Ku80 antibody (Fig. 1B). To further confirm the identity of the protein recognized by these two antibodies, reciprocal immunodepletion experiments were carried out. Preclearing of whole cell lysates with the HAL-1/13 antibody removed all antigen reactive with the anti-Ku80 antibody and vice versa (Fig. 1C). Immunostaining of HeLa cells showed that the Ku80 antigen is expressed on the cell surface and that the HAL-1/13 MAb also stains the surface of HeLa cells (Fig. 1D). Cell surface immunofluorescence analysis using two other commercially available α -Ku80 antibodies (N9C1 and S10B1) confirmed expression of Ku80 on the plasma membrane (data not shown). In previous studies using isotype-matched MAbs (IgG2a) as controls, we have demonstrated that immunoprecipitations of 125 I- or 35 S-labeled total cellular proteins with the HAL-1/13 antibody gave only two products, Ku80 and its heterodimeric partner, Ku70, demonstrating the specificity of the antibody (21).

Ectopic expression of the cDNA encoding the HAL-1/13-Ku80 antigen confers adhesion to lymphoid and myeloid cells. The HAL-1/13 antigen was initially described as an adhesion molecule and the Ku antigen as

a multifunctional nuclear effector. The unexpected identity of these two molecules suggested a new role for Ku antigen as a surface adhesion receptor. To verify and elucidate the adhesion properties of HAL-1/13-Ku80, murine NIH/3T3 fibroblasts were transfected with the HAL-1/13-Ku80 cDNA. If Ku antigen can indeed function as an adhesion molecule, then transfection of fibroblasts should result in surface expression of HAL-1/13-Ku80, which would confer to fibroblasts the ability to adhere to human lymphoid cells. Murine fibroblast NIH/3T3 cells were chosen as the background for ectopic expression of HAL-1/13-Ku80 because the Ku antigens are expressed only at very low levels in nonprimate cells, and the antibodies against primate Ku antigens are not cross-reactive with murine Ku proteins (67). We have also demonstrated that neither the anti-HAL-1/13 nor the anti-Ku antibodies cross-reacted with any NIH/3T3 cell surface or intracellular antigens (Fig. 2).

The HAL-1/13-Ku80 gene (PRNZ) was cloned into an expression vector (pCINeo), and the resulting vector (PRNZ-1) or the empty pCINeo vector alone was transfected into NIH/3T3 cells, followed by selection in G418. G418-resistant clones, or pools of clones, were assayed for expression of HAL-1/13-Ku80. Immunoprecipitation of whole cell extracts from these clones, using an anti-Ku80 antisera, yielded an 80-kDa protein that was immunoreactive with the HAL-1/13 antibody. Parental or control-transfected NIH cell lines (NIH pCINeo) were negative for this protein (Fig. 3A). Clones that expressed HAL-1/13-Ku80 were assayed for cell surface expression of HAL-1/13-Ku80, using the HAL-1/13 antibody (Fig. 3B) or the Ku80 antibody (not shown). All HAL-1/13-positive clones were also Ku80 positive by cell surface staining (not shown), again demonstrating the identity of HAL-1/13 and Ku80. In

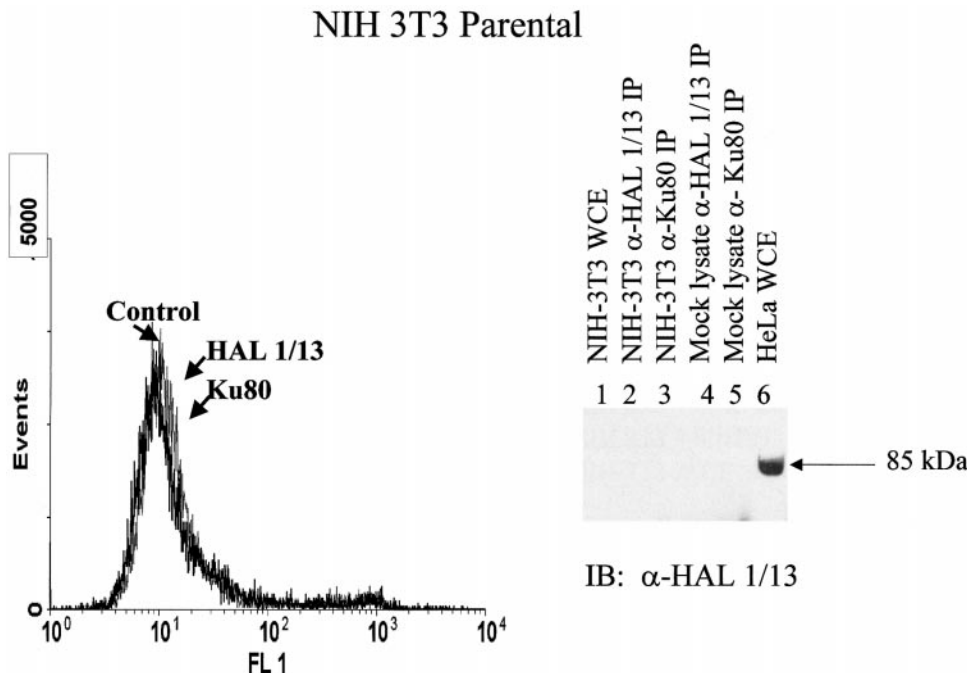
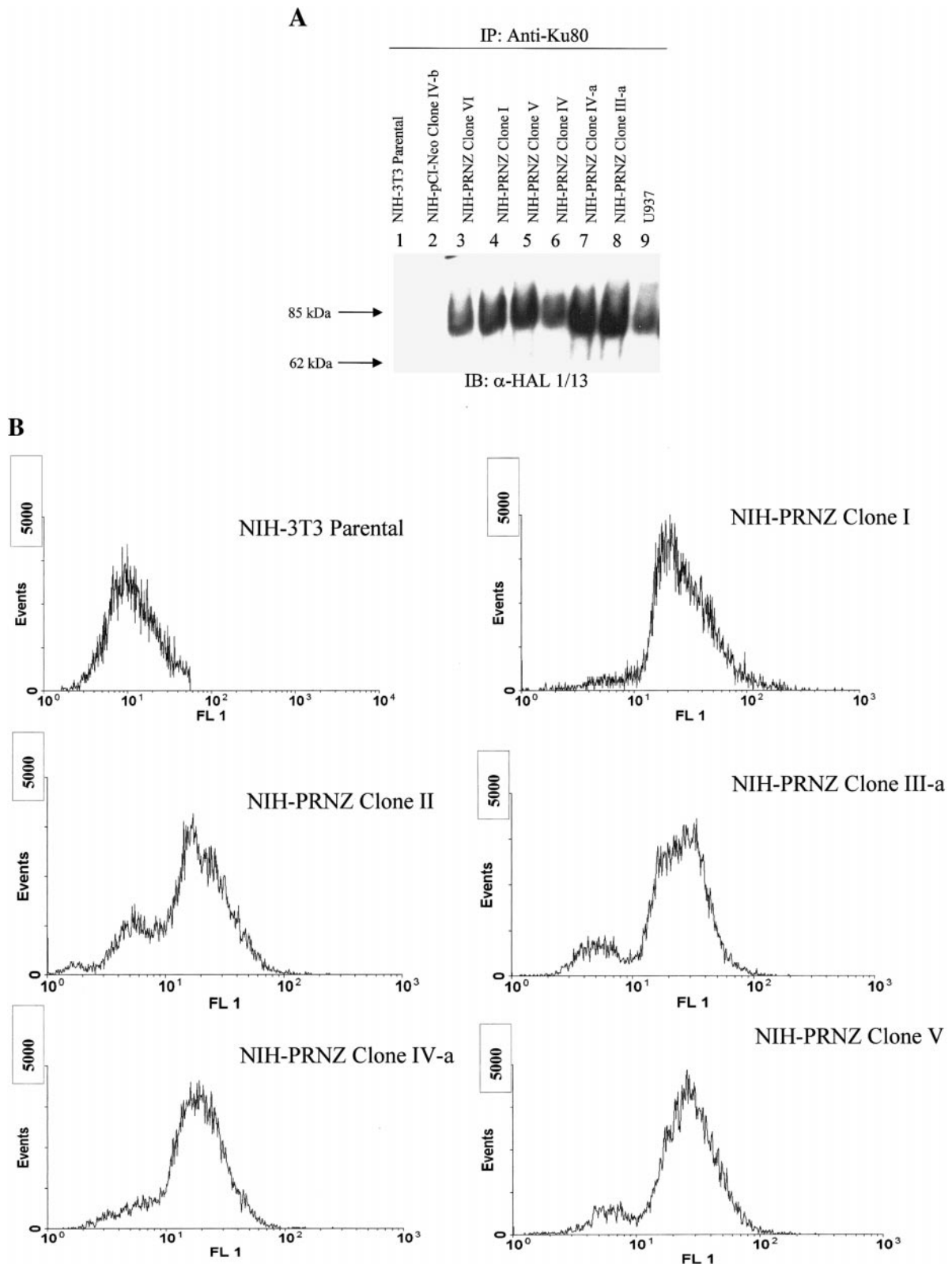


Fig. 2. Lack of expression of HAL-1/13-Ku80 protein in NIH cells. NIH cells were stained with the undiluted HAL-1/13 MAb hybridoma supernatant or an α -Ku80 MAb (Mab 111) diluted 1:100 (Ku80) or with no primary antibody (control), and then with an FITC-conjugated sheep (Fab')₂ anti-mouse IgG. Flow cytometric profiles are shown (left). Total cellular proteins from NIH cells (NIH/3T3 WCE) or HeLa cells (HeLa WCE) were left untreated or immunoprecipitated with MAbs directed against HAL-1/13 (NIH/3T3 anti-HAL-1/13 IP) or Ku80 (NIH/3T3 anti-Ku80 IP) and separated by 7.5% SDS-PAGE. As negative controls, the HAL-1/13 MAb preparation (mock lysate anti-HAL-1/13 IP) and a Ku80 MAb preparation (mock lysate anti-Ku80 IP) in PBS/10% serum were also separated. After transfer, the immunoprecipitated products were immunoblotted with the HAL-1/13 MAb and developed as described in the legend to Fig. 1. The autoradiograms are shown here. The migration position of an 85-kDa molecular mass marker is shown.

addition, all clones that expressed HAL-1/13-Ku80 by immunoblotting also expressed this antigen on the surface.

We next determined if this ectopically expressed HAL-1/13-Ku80 antigen could serve as a functional

adhesion receptor. The adhesion of the NIH parental line, control-transfected cells (NIH pCINeo), and the HAL-1/13-expressing clones to three types of hematopoietic cell lines was examined (Fig. 4). JY is an EBV-immortalized peripheral blood B lymphocyte line (39),



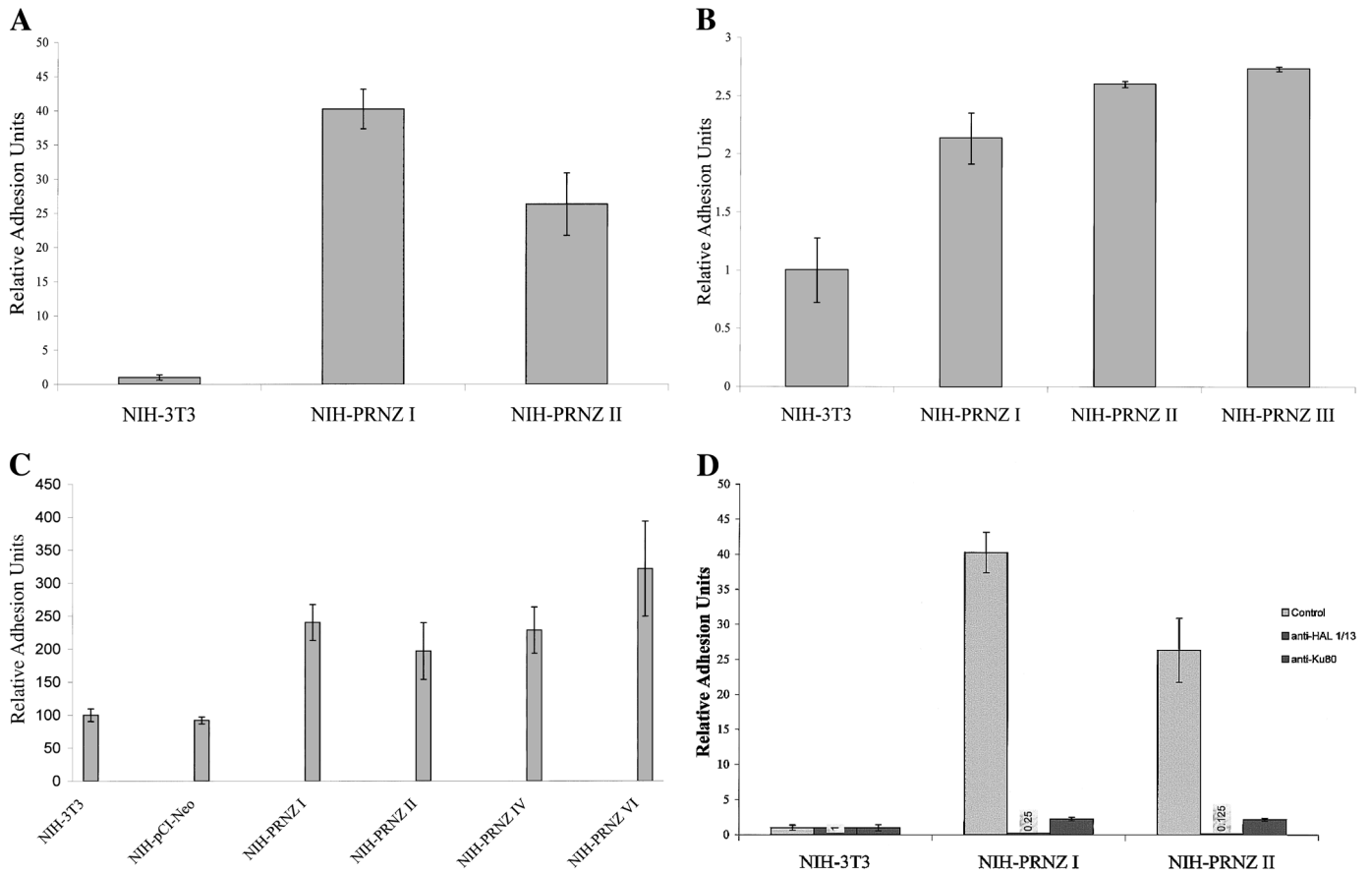


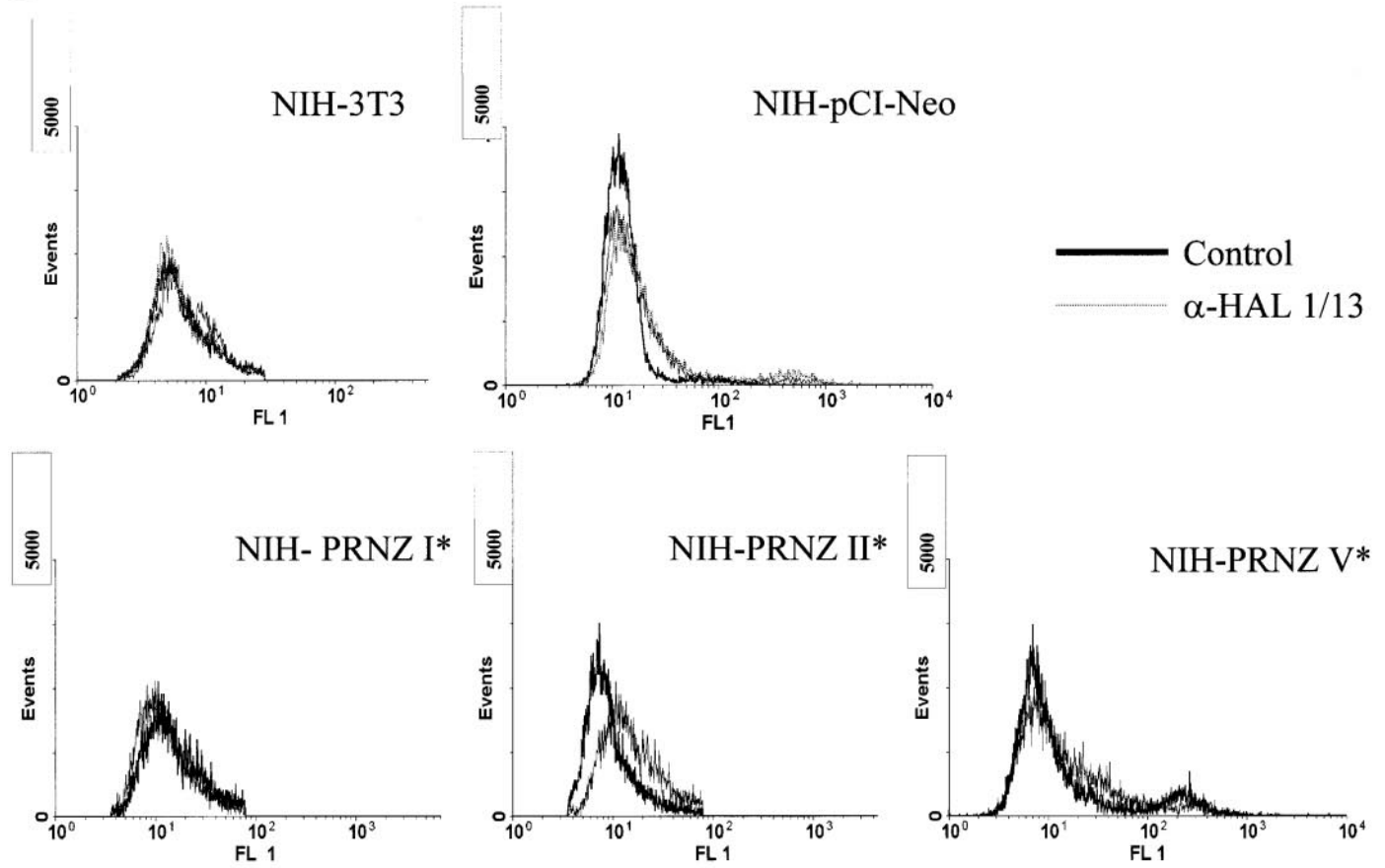
Fig. 4. Adhesion of human lymphoid cell lines cells to 3T3 cells ectopically expressing HAL-1/13-Ku80. Untransfected parental NIH/3T3 cells, control-transfected cells, NIH-pCI-Neo, and NIH/3T3 cells transfected with PRNZ (NIH-PRNZ clones I, II, III, IV, and VI) were used in adhesion experiments with fluorescence-tagged JY cells (A), Jurkat cells (B), and U-937 cells (C), as described in MATERIALS AND METHODS. Adhesion of each cell line to the parental NIH/3T3 cells was arbitrarily given a value of 1 adhesion unit. Error bars represent the SD of the mean taken from 3 experiments, each performed in triplicate. D: adhesion of JY cells to NIH/3T3 PRNZ-transfected cells is abrogated by both the HAL-1/13 MAb and α -Ku80 MAb. NIH/3T3 cells and NIH-PRNZ clones I and II were pretreated with either the HAL-1/13 or the α -Ku80 mAb (Mab 111) or with W6/32 (an IgG2a isotype-matched control MAb; Control), as described in MATERIALS AND METHODS, and cell adhesion studies were carried out in parallel with the studies shown in A. Error bars represent the SD of the mean of 3 experiments, each performed in triplicate.

Jurkat is a T lymphoblastoid cell line (68), and U-937 is a line derived from a histiocytic lymphoma with myelomonocytoid characteristics (62). As expected, the adhesion of each of the lymphoid lines for parental or control-transfected lines was quite low, because NIH/3T3 fibroblasts are not known to express any adhesion molecules capable of interacting with human lymphocytes. Adhesion of JY, Jurkat, or U-937 cells to control-transfected NIH clones (NIH pCINeo) was always

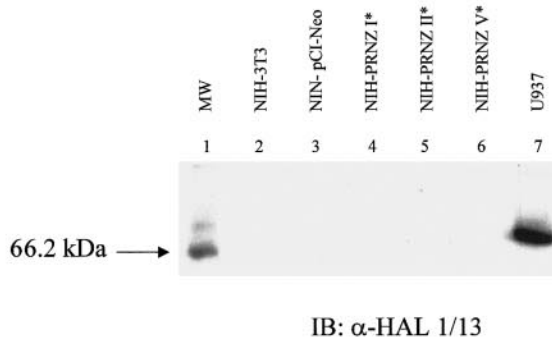
equivalent to the adhesion observed to untransfected, parental NIH/3T3 cells. Ectopic expression of HAL-1/13-Ku80 on the surface of NIH cells increased the binding of JY cells 40- and 28-fold (PRNZ clones I and II, respectively; $P < 0.001$; Fig. 4A). The binding of Jurkat cells was enhanced less, but still significantly, by expression of HAL-1/13-Ku80, with relative increases in adhesion ranging from 2.2- to 2.8-fold ($P < 0.02$), according to the particular clone of NIH trans-

Fig. 3. Expression of human HAL-1/13-Ku80 protein in NIH/3T3 transfectants. A: total cellular proteins from NIH/3T3 cells (NIH/3T3 Parental), from U-937 cells, from NIH cells transfected with the empty vector pCI-Neo (NIH-pCI-Neo Clone IV-b), or from the PRNZ-transfected NIH cell clones VI, I, V, IV, IV-a, and III-a were immunoprecipitated with an α -Ku80 MAb (Mab 111). The immunoprecipitated proteins were separated by 7.5% SDS-PAGE, transferred to a membrane, and immunoblotted with the HAL-1/13 MAb. The blots were developed with an ECL reagent, and the film images produced are shown here. The migration positions of 85- and 62-kDa molecular mass markers are shown. The HAL-1/13-Ku80 protein detected in the U-937 cell lysate (lane 9) serves as a positive control. B: NIH/3T3 cells transfected with the empty pCI-Neo vector (NIH/3T3 Parental) or clones of NIH/3T3 cells transfected with the PRNZ expression vector (NIH-PRNZ clones I, II, III-a, IV-a, and V) were stained with the anti-HAL-1/13 MAb and then with an FITC-conjugated sheep (Fab')₂ anti-mouse IgG as the secondary antibody. Fluorescent flow cytometric profiles are shown.

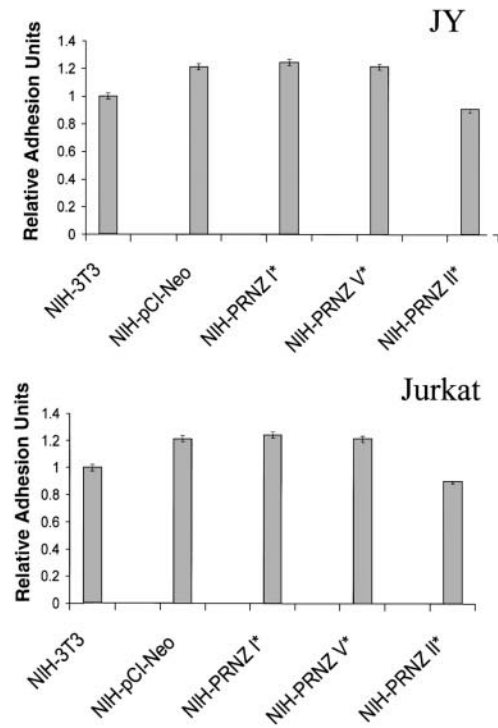
A



B



C



fectant studied (PRNZ clones I and III, respectively; Fig. 4B). HAL-1/13-Ku80 expression also enhanced the relative binding of U-937 cells to the transfected NIH cell 2- to 3.3-fold (PRNZ clones II and VI, respectively; $P < 0.05$; Fig. 4C).

To confirm that the enhanced adhesion of human cells to the HAL-1/13-transfected murine NIH/3T3 cells was mediated through HAL-1/13-Ku80, we attempted to block this adhesion using either the HAL-1/13 MAb or an anti-Ku80 MAb. Each antibody completely blocked adhesion of JY cells to the NIH cell clones expressing HAL-1/13-Ku80 (by ~200-fold for the HAL-1/13 MAb and 10- to 20-fold for the anti-Ku80 MAb), reducing adhesion to control cell line adhesion levels ($P < 0.001$; Fig. 4D). Neither antibody had any significant effect on JY cell binding to control NIH/3T3 cells. Both antibodies also completely abrogated the enhanced adhesion of Jurkat and U-937 cells to the HAL-1/13-transfected NIH/3T3 cells (data not shown).

Over time, the PRNZ-transfected clones were observed to invariably lose cell surface expression of HAL-1/13, despite continued culture in the presence of G418 (clones that lost expression are designated by an asterisk after the name of the clone; Fig. 5A). Parallel studies using an anti-Ku80 MAb for staining verified the loss of Ku80 antigen on the surface (not shown). Loss of cell surface expression of HAL-1/13-Ku80 expression was accompanied by loss of intracellular expression of the transfected gene product (Fig. 5B). The effect of this loss of HAL-1/13-Ku80 expression on the cell-cell adhesive properties of the transfected clones was assessed. Adhesion of JY or Jurkat cells to these loss-of-expression variant clones was dramatically inhibited or totally abrogated (compare the relative adhesion of Jurkat and JY to the respective PRNZ clones in Fig. 4, A and B).

Effect of hypoxia on cell surface expression of HAL-1/13-Ku80 and its association with Ku70. The HAL-1/13 antibody was initially selected for its ability to block adhesion of leukocytes to hypoxic endothelium and RD cells (21). More recently, we have shown that this antibody can inhibit invasion of hypoxic neuroblastoma and breast carcinoma cells in vitro and that hypoxia increases expression of HAL-1/13 on the surface of these cells (18). Having ascertained that HAL-1/13 is identical to the Ku80 antigen and that the Ku antigen is predominantly expressed in the nucleus, we investigated whether hypoxic treatment affects the distribution of HAL-1/13 be-

tween the nucleus and plasma membrane. U-937 and Jurkat cells, both of which express significant levels of HAL-1/13 on their surface under normoxic conditions, were chosen for these experiments. Forty-eight hours of hypoxic ($P_{O_2} = 20$ Torr) exposure of U-937 cells resulted in a greater than fivefold increase in cell surface expression of the HAL-1/13-Ku80 antigen, as determined using a MAb specific for each for staining (Fig. 6A). Shorter periods of hypoxic exposure (24 h) did not result in consistent induction in U-937 cells (not shown). Hypoxic conditions also induced HAL-1/13-Ku80 antigen expression on Jurkat cells, although to a lesser extent (~2.5-fold), and induction of expression on these cells was apparent at 24 h (Fig. 6B) and did not increase further at 48 h (not shown).

Ku80 in the nucleus is associated with Ku70. Ku70 expression on the surface has also been reported in certain cell lines. To determine whether hypoxia also upregulates Ku70 or HAL-1/13-Ku80/Ku70 heterodimers on the cell surface, U-937 cells were stained with either a MAb recognizing Ku70 or an antibody recognizing the Ku70/80 complex (67). U-937 cell membranes were strongly positive for each antigen, but levels of Ku70 or Ku70/80 complex expression did not increase in response to hypoxia (Fig. 6C), suggesting that not all of the Ku80 protein induced on the cell surface by hypoxia is associated with Ku70.

Intracellular redistribution of HAL-1/13-Ku80 by hypoxia. Potential mechanisms for hypoxic induction of cell surface expression of HAL-1/13-Ku80 include increases in the total cellular levels of HAL-1/13-Ku80 or redistribution of intracellular HAL-1/13-Ku80 to the membrane. Isolated or pooled subcellular fractions (nuclear fractions and plasma membrane fractions) of U-937 cells were assayed for relative levels of Ku80, with or without exposure to hypoxic conditions. Because cytoplasmic fractions of U-937 cells contained no detectable Ku80 or Ku70 (data not shown), the nuclear and membrane fractions were pooled to provide an estimate of total cellular levels of the protein. Immunoblotting with a Ku80 MAb (Fig. 7A) showed no significant changes in total cellular HAL-1/13-Ku80 after hypoxic exposure. Levels of HAL-1/13-Ku80 in the nucleus, relative to the plasma membrane, were increased in membranes relative to the nucleus under hypoxic conditions, with a nuclear-to-membrane (N:M) ratio of 2.3:1 under normoxic conditions, changing to 1:2.9 under hypoxic conditions. Parallel experiments

Fig. 5. Coincident loss of HAL-1/13-Ku80 antigen expression and of adhesive properties in the antigen NIH-PRNZ transfectants. A: cells were stained with either anti-HAL-1/13 MAb and FITC-conjugated sheep (Fab')₂ anti-mouse IgG (broken line) or FITC-conjugated sheep (Fab')₂ anti-mouse IgG alone (Control, solid line). Cell surface expression of the HAL-1/13 antigen was analyzed by fluorescence-activated cell sorter (FACS). Cell lines analyzed include parental NIH/3T3 cells, NIH-pCI-Neo as a transfection control, and NIH-PRNZ clones I*, II*, and V*. B: total cellular protein lysates were separated on 10% SDS-PAGE, transferred to a membrane, and immunoblotted with the anti-HAL-1/13 MAb. The blots were developed with an ECL reagent, and the autoradiograms are shown. Cell lines analyzed include parental NIH/3T3 cells, NIH-pCI-Neo as a transfection control, NIH-PRNZ clones I*, II*, and V*, and U-937 as a positive control for Ku80 detection. The migration position of the molecular mass marker of 66.2 kDa is shown (MW). C: adhesion of JY cells (top) and Jurkat cells (bottom) to parental NIH/3T3 cells, transfection control cell line NIH-pCI-Neo, and the NIH-PRNZ clones I*, V*, and II*, which had lost cell surface expression of the HAL-1/13-Ku80 antigen. Error bars represent the SD of the mean of 3 experiments, each in triplicate.

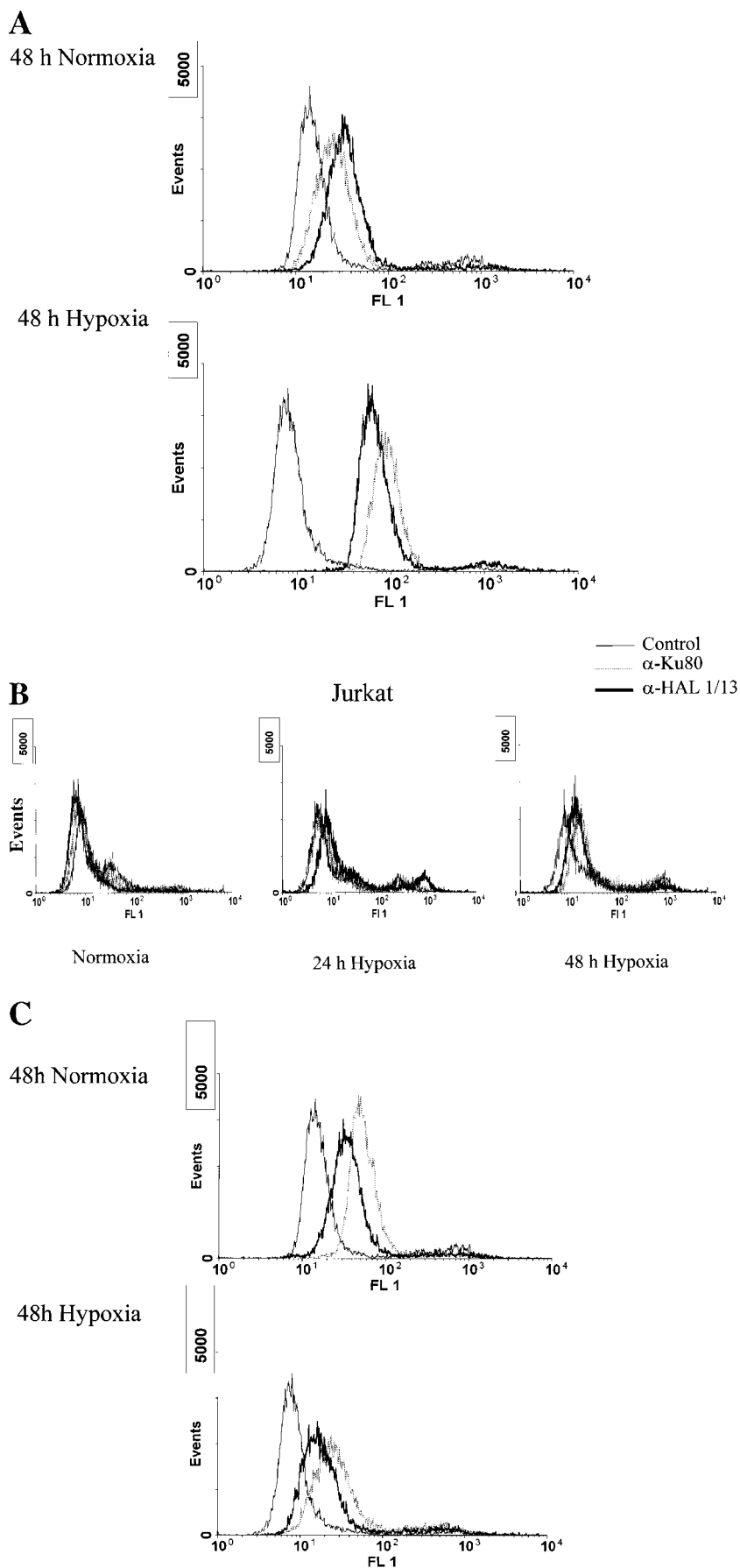


Fig. 6. Hypoxia-induced cell surface expression of HAL-1/13 and Ku antigen subunits. U-937 (A) or Jurkat cells (B) were exposed to normoxic or hypoxic environments for 24 or 48 h and stained with either HAL-1/13 (heavy line) or α -Ku80 (Mab 111; dashed line) MAbs, or with FITC-conjugated sheep (Fab')₂ anti-mouse IgG (Control) alone. Cell surface expression of the HAL-1/13 antigen was analyzed by FACS. C: U-937 cells were exposed to normoxic or hypoxic environments for 48 h and stained with anti-Ku70 (heavy line), anti-Ku70/80 (dashed line) MAbs, or with FITC-conjugated sheep (Fab')₂ anti-mouse IgG (Control) alone. Cell surface expression of HAL-1/13 antigen was analyzed by FACS.

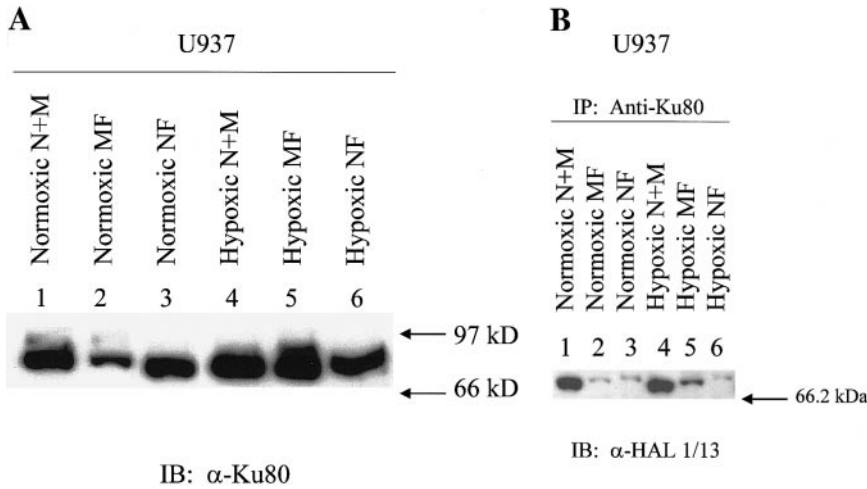


Fig. 7. Levels of HAL-1/13-Ku80 antigen in nuclear or membrane compartments as a function of oxygen tension. Twenty micrograms of protein from nuclear fractions (NF) or membrane fractions (MF) obtained from U-937 cells under normoxic conditions or exposed to hypoxia for 48 h, or 20 μ g of pooled (N+M, 1:1) fractions, were separated on 7.5% SDS-PAGE, transferred to a membrane, and immunoblotted with the HAL-1/13 MAb (A). Alternatively, 20 μ g of protein from the same cell fractions or 40 μ g of pooled N+M fractions were first reacted with the α -Ku80 MAb (Mab 111), and the immunoprecipitated proteins were separated on 7.5% SDS-PAGE, transferred to a membrane, and immunoblotted with the α -HAL-1/13 MAb (B). The blots were developed with an ECL reagent, and the autoradiograms are shown. The migration positions of 97- and 66-kDa molecular mass markers are indicated.

using the HAL-1/13 MAb yielded identical results (not shown). This same hypoxia-induced shift in subcellular distribution was observed when an anti-Ku80 MAb was used to first immunoprecipitate proteins from the whole cell lysates or subcellular fractions, followed by separation and immunoblotting with the anti-HAL-1/13 MAb (N:M ratio of 1.6:1 for normoxia shifting to 1:3.6 for hypoxia; Fig. 7B). The reciprocal of this latter experiment (immunoprecipitating with the HAL-1/13 MAb and immunoblotting with an anti-Ku80 MAb) gave identical results (not shown).

In contrast, the levels of Ku70 in the membrane fraction relative to levels in the nuclear fraction did not

increase during hypoxic exposure of U-937 cells and instead showed a modest, but consistent, decrease (N:M ratio of 1:1.9 for normoxia shifting to 2.2:1 for hypoxia), while total cellular levels of Ku70 increased somewhat (Fig. 8A, right). The amounts of Ku70 that could be coimmunoprecipitated with HAL-1/13-Ku80 showed a similar and consistent pattern of relative decrease in membrane fractions after hypoxia (N:M ratio of 2.2:1 for normoxia and 4.2:1 for hypoxia; Fig. 8A, left). A reciprocal immunoprecipitation using an MAb directed against Ku70 and separation on a denaturing gel, followed by probing of the immunoprecipitated, complexed proteins with the anti-HAL-1/13 MAb

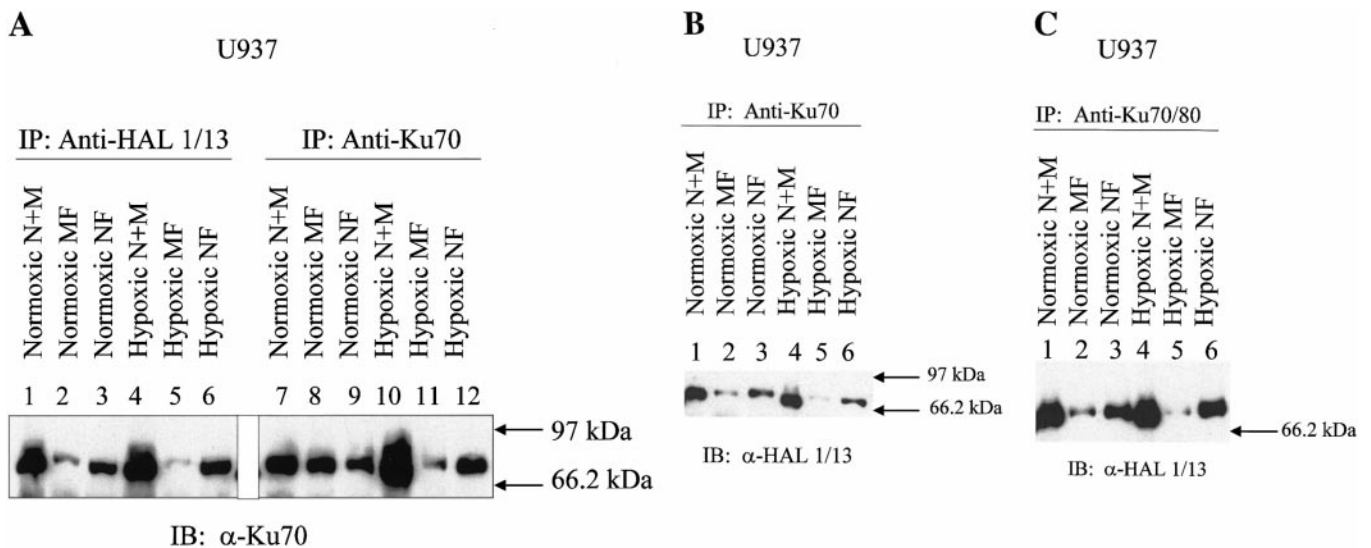


Fig. 8. Association of HAL-1/13-Ku80 antigen and Ku70 antigen in subcellular compartments as a function of oxygen tension. A: 20 μ g of protein from NF or MF obtained from U-937 cells under normoxic conditions or exposed to hypoxia for 48 h or 40 μ g of pooled N+M fractions (1:1) were reacted with the HAL-1/13 MAb (left) or an α -Ku70 MAb (Mab H3H10; right), and the immunoprecipitated proteins were separated on 7.5% SDS-PAGE, transferred to a membrane, and immunoblotted with the α -Ku70 MAb (Mab H3H10). Alternatively, 20 μ g of protein from the same cell fractions or 40 μ g of pooled N+M fractions were first reacted with an α -Ku70 MAb, and the immunoprecipitated proteins were separated on 7.5% SDS-PAGE, transferred to a membrane, and immunoblotted with the HAL-1/13 MAb (B). Alternatively, 20 μ g of protein from the same cell fractions or 40 μ g of pooled N+M fractions were first reacted with an α -Ku70/80 MAb (Mab 162), and the immunoprecipitated proteins were separated on 10% SDS-PAGE, transferred to a membrane, and immunoblotted with the HAL-1/13 MAb (C). The blots were developed with an ECL reagent, and the autoradiograms are shown. The migration positions of 97- and 66-kDa molecular mass markers are indicated.

(Fig. 8B) or an anti-Ku80 MAb (not shown) also showed less association of HAL-1/13-Ku80 with Ku70 in the membrane fraction during hypoxia (a 48% decrease), whereas association of Ku70 and HAL-1/13-Ku80 in the nuclear fraction did not change. A parallel experiment using the anti-Ku70/Ku80 complex-specific MAb for immunoprecipitation confirmed the relative decrease (a 57% decrease) in Ku70/Ku80 complex formation on the cell membrane during hypoxic exposure (Fig. 8C). Thus despite the significant increases in membrane-localized Ku80 in response to hypoxia, there is no evidence for a comparable increase in cell surface Ku70 expression or in Ku70/80 complex formation.

DISCUSSION

The nuclear localization and nuclear functions of Ku, and more specifically the Ku80 component, have been well characterized by both cell fixation and immunocytochemical techniques (26, 53, 54, 67, 70, 71). Ku, predominantly as the Ku70/Ku80 heterodimer, is dispersed in particulate fashion throughout the nucleus. During mitosis, Ku is concentrated at the nuclear periphery in interphase cells and associated with metaphase chromosomes (26). Ku nuclear staining displays a reticular pattern with sparing of nucleoli (41), although Ku is associated with nucleoli at certain phases of cell cycle (71). Ku80 has been isolated from nucleoli and active (DNase sensitive) chromatin (70). A wide variety of functions has been reported for the nuclear component of Ku, including DNA double-stranded break repair and V(D)J recombination (4, 5, 24, 36, 37, 59), DNA replication (see Ref. 2 for review), DNA transcription (see Ref. 33 for review), ATP-dependent helicase activity (65), DNA-dependent ATPase activity (7), stimulation of elongation property of RNA polymerase II (14), binding to human immunodeficiency virus-1 transactivating region RNA (31), maintenance of normal telomere length in yeast (23, 34, 45, 48), and chromatin condensation in G_2/M (44), among others.

Although the nuclear-localized fraction of the Ku80 protein has been predominantly studied in connection with DNA repair and replication, independent observations demonstrate that Ku80 can also be a cell surface protein (13, 28, 49) and a possible participant in signal transduction (35) and cell-cell interactions (63). Analysis of the predicted amino acid sequence of the Ku80 cDNA identified a large hydrophobic region near the NH_2 terminus of the molecule (72). Yet, even if this region represents a membrane-spanning domain, it remains unclear why all primate cells that express Ku80 in the nucleus do not also express it on the surface. Indeed, in limited surveys of the normal tissue distribution of Ku80, we have found cell surface expression of Ku80 only on endothelial cells. In contrast, the HAL-1/13-Ku80 antigen is found on the surface of many leukemic and solid tumor cells and cell lines, including T and B lymphomas, myeloid leukemias, neuroblastoma, RD, and breast carcinoma cells (this report and Refs. 18 and 21). Others have reported that

resting (G_0) but not proliferating lymphocytes are positive for cell surface expression of Ku antigen (1). It is interesting to note that, in our studies, every transfected NIH/3T3 clone that expressed HAL-1/13-Ku80 in the nucleus also expressed the antigen on the cell surface, suggesting that the lack of expression of Ku80 on the surface of most normal human cells, and its frequent expression on tumor cells, may be an active restriction against transport or expression rather than the result of uncharacterized, aberrant membrane transport processes in tumor cells.

Although Ku70 has also been reported to be expressed on the surface of some (tumor) cells and also possesses several predicted hydrophobic domains, at least one of which is large enough to span the membrane (8, 49), our data suggest that it is not likely that Ku70 targets Ku80 to the membrane, or vice versa. We could demonstrate some association of Ku70 and Ku80 on the cell surface by coimmunoprecipitation studies. In addition, an antibody specific for the Ku70/Ku80 heterodimer showed reactivity with membrane components of certain cells. [This latter antibody is known to recognize a conformational epitope, depending on the quaternary structure of Ku (67). Our unpublished studies indicate that the epitope recognized by this antibody actually resides on the Ku70 molecule.] However, induction of cell surface expression of Ku80 by hypoxia did not produce a commensurate increase in Ku70 antigen levels, or in Ku70/Ku80 complexes, at the cell membrane. Furthermore, cell surface expression of Ku70 and Ku80 is dissociated in certain tumors (unpublished observations), and independent regulation or translocation of the subunits of Ku has been described (16, 32). Posttranslational modifications of Ku80 [serine phosphorylation by DNA-PK (7, 29) and tyrosine phosphorylation, possibly by CD40 (43)] have been reported, but our preliminary studies have found no evidence of differences in the phosphorylation state of Ku80 on the cell surface compared with the nuclear-localized fraction.

In the cell lines examined in this report, exposure to hypoxia was marked by a reversal in the ratios of nuclear-associated to membrane-associated Ku80, without changes in the total cellular amounts of Ku80. Furthermore, HAL-1/13-Ku80 transcript levels in endothelial cells do not change in response to hypoxia (unpublished observations). Thus the induction of cell surface expression of Ku80 by hypoxia may be the result of redistribution among cellular compartments rather than of new synthesis. Translocation of Ku80 from the cytosol to the nucleus after treatment of a cell line with somatostatin has been reported (64). Intracellular redistribution of Ku70 and Ku80 from the nucleus to the cytoplasm as a function of cell density or confluence has also been described (16), and apparent translocation from the cytoplasm to the cell surface has been observed in some myeloma cell lines after stimulation with CD40 ligand (63). Translocation of Ku80 from the cytoplasm to the nucleus has been observed after stimulation through CD40 in B cell lines (43), and Ku80 dissociates from chromosomes during mitosis

(71). Although there does not appear to be a transcriptional component to the induction of cell surface expression of Ku80 we observed, Ku gene transcription is thought to be regulated by cell cycle, being activated in late G₁ (71), and modulation of the total cellular levels of Ku in response to phorbol 12-myristate 13-acetate, calcium signals, or serum have led to the proposal that Ku may function as a sensor of the cellular environment (50, 61).

Hypoxic exposure resulted in no induction of HAL-1/13 expression on HAL-1/13-Ku80-transfected NIH/3T3 cells (unpublished observations). Although it is possible that this was because the NIH/3T3 transfectants were already expressing "maximal" levels of HAL-1/13-Ku80 on the plasma membrane, the fact that we selected for transfectants expressing a range of levels of expression, with none expressing higher levels than any of the human cell lines we have examined spontaneously express, make this unlikely. Interestingly, there appears to be a selection against expression of Ku80 in these murine cells, with every independently isolated murine clone losing expression within 6 wk, despite continuous selection in G418.

Ectopic expression of HAL-1/13-Ku80 in murine cells was used to assay for a direct role of Ku80 as an adhesion molecule. Murine fibroblasts have often been used as a neutral or null background for the study of ectopic expression of human adhesion molecules because of the very low background binding of human leukocytes to these cells. In general, adhesion molecules appear to be poorly conserved between primate and rodent species. This is also the case with Ku80, where there is little or no antigenic conservation between murine and human (47). HAL-1/13-Ku80 transfectants, which expressed levels of human Ku80 comparable with those expressed on human cells were isolated to avoid the potentially confounding effects of nonphysiological overexpression. Ectopic cell surface expression of HAL-1/13-Ku80 antigen invariably conferred increased adhesiveness of NIH cells, both clones and pools, to three different lymphoid cell lines, providing direct evidence for the ability of Ku80 to function as an adhesion molecule. Furthermore, spontaneous loss of expression of HAL-1/13 correlated with loss of adhesive properties, thereby strengthening the correlation. Finally, MAbs against HAL-1/13-Ku80 attenuated the adhesion, in agreement with other studies that have indirectly suggested a role for HAL-1/13-Ku80 as an adhesion molecule (21, 63).

Although ectopic expression of HAL-1/13-Ku80 in NIH/3T3 cells conferred increased adhesiveness to three different human lymphoid cell lines, the magnitude of the adhesion varied, with JY cells adhering best and Jurkat and U-937 less strongly. The reason for these differences in adhesion level might be related to differing levels of the counterreceptor/ligand for HAL-1/13-Ku80 on these cells. Alternatively, it may be relevant that JY cells do not express HAL-1/13, whereas Jurkat and U-937 cells have high levels of HAL-1/13 on the cell surface, and some type of competitive process may be operative. The counterreceptor/ligand for Ku80

has not yet been determined. We have presented evidence that neutralizing antibodies directed against lymphocyte functions-associated antigens (both the CD11a and the CD18 subunits) can partially block hypoxia-induced, HAL-1/13-dependent adhesion (21). A ligand-dependent association of CD40 and cytoplasmic Ku70/80 has been reported, but this appears to be mediated through an intracytoplasmic domain of CD40 (43). Homotypic Ku-Ku interactions are also a formal possibility. In our previous studies (21), both the adhering cells and the adherent cells expressed HAL-1/13-Ku80 on their surfaces. Pretreatment of either cell adhesion partner with the HAL-1/13 MAb prevented adhesion, but a nonspecific steric effect could not be completely ruled out in those experiments. In the current studies, the presence of HAL-1/13-Ku80 on Jurkat and U-937 cells did not augment their adhesion to Ku80-transfected NIH/3T3 cells, compared with the HAL-1/13-Ku80-negative JY cells, arguing indirectly against homotypic Ku80-Ku80 interactions. Ku70 and Ku80 can heterodimerize (53, 55, 72). The coimmunoprecipitation studies described herein demonstrate a constitutive level of association of Ku70 and Ku80 on the cell surface, but this likely represents association of molecules on the same cell, rather than intercellular associations. Furthermore, anti-Ku70 antibodies do not disrupt Ku80-dependent cell-cell adhesion (although it is also possible that these MAbs may not recognize potentially functional epitopes on Ku70). Studies are underway to determine what other cell surface protein might associate with Ku80 during cell-cell adhesive interactions.

The physiological significance of HAL-1/13-Ku80-mediated cell-cell adhesion is as yet undetermined. Although we have clearly demonstrated that Ku80 mediates the increased adhesion of leukocytes to endothelium under hypoxic conditions (21), whether deficiency in Ku80 might lead to impairment of normal immune function through abrogation of an immune cell-cell adhesion pathway is difficult to assess because mice deficient in Ku80 or Ku70 lack normal B cell or B and T cell development (reviewed in Refs. 10 and 15). There is evidence, however, suggesting that HAL-1/13-Ku80-mediated cell-cell adhesion may play a role in pathological processes such as tumor invasion. Although the HAL-1/13 antigen is not present on most normal lymphoid cells, many leukemic cell lines, including Jurkat, Molt-4, HL-60, and U-937, react strongly with the anti-HAL-1/13 antibody (21). We have also recently shown that hypoxia induces cell surface expression of HAL-1/13-Ku80 on a number of solid tumor cell lines, including neuroblastoma (Kelly, SY-SK), breast carcinoma (MCF7), and RD cells (18, 21). When these cells are exposed to low-oxygen environments, their ability to invade endothelial cell monolayers and to transmigrate through Matrigel-coated filters is increased with, coincident with, and dependent on, increased cell surface expression of HAL-1/13-Ku80. Hypoxia is known to enhance the metastatic potential and invasiveness of tumor cells (11, 12, 27, 56, 57, 60, 74, 75). Together, these findings suggest

that HAL-1/13-Ku80 may play a major role in regulating the invasive potential of tumor cells, as well as mediating leukocyte-endothelial cell interactions.

This work was supported by National Cancer Institute (NCI) Public Health Service Grant CA-50459, by a Department of Defense Breast Cancer Research grant, and by a Leukemia Society of America grant (to D. V. Faller). E. M. Lynch was supported by NCI Oncobiology Training Grant T32-CA-64070.

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