Novel Cell Cycle-related Nuclear Proteins Found in Rat and Human Cells with Monoclonal Antibodies

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ABSTRACT

Monoclonal antibodies which react specifically with the nuclei of interphase cells recognized three nuclear antigens with molecular weights of 110,000 (p110), 85,000 (p85), and 18,000 (p18). p110 and p85 were found in eight tumor cell lines but were not found in resting lymphocytes. p18 was found in resting lymphocytes as well as the tumor cell lines. Protein p85 appeared in phytohemagglutinin-stimulated lymphocytes in the G1 phase and protein p110 appeared in the S phase. p110 and p85 were localized to the extranucleolar chromatin while p18 was distributed throughout the nucleus and was determined by microscopic and DNA digestion studies to be DNA associated. The anti-p110 antibody recognized a component of the DNA polymerase α complex. Three novel nuclear proteins were identified using monoclonal antibodies. Two of these proteins (p110 and p85) are proliferating cell nuclear and nucleolar antigen-like while the third (p18) is not cell cycle dependent.

INTRODUCTION

The G1-S phase of the cell cycle is crucial in determining whether the cell proceeds from the resting G0 state to initiate DNA synthesis and becomes committed to cell division (1, 2). The precise mechanisms which permit cell proliferation to proceed beyond the "restriction point(s)" are not known, but it is suggested that an accumulation of "trigger proteins" must occur to continue the mitogenic cascade (3). If such an accumulation of specific proteins occurs, then phase-specific proteins should be demonstrable in the G1-S phase.

Phase-specific PCNA were detected with autoimmune sera during the G1-S phase of the cell cycle (4-9). With two-dimensional gel electrophoresis, the protein referred to as cyclin (M, 36,000/pI 5.0) was found to be a PCNA which appears at the G1-S interface (10-12). Further studies have shown that the synthesis of cyclin correlates directly with the proliferative state of normal and transformed cells (13). Celis et al. (14) have shown that some of these autoimmune sera react specifically with cyclin. Celis et al. (13) suggest that cyclin may be important in the identification of blast cells in leukemia patients and in determination of nuclear synchrony.

Busch et al. (15, 16) have developed rabbit polyclonal antiserum to nucleolar proteins. These antisera were shown to distinguish malignant tissues from normal, resting tissues suggesting that some nucleolar antigens may be associated with cell growth (15, 16). Polyclonal antisera generated against a spectrum of HeLa proteins revealed changes in the expression of particular proteins during the cell cycle (17). Bhorjee et al. (18) developed five monoclonal antibodies which exhibit a cell cycle-dependent distribution pattern; the molecular weights of these proteins were not reported.

Monoclonal antibodies to other PCNA-like antigens have recently been reported including, a M, 86,000 nuclear protein (12, 19) and a nuclear protein recognized by the Ki 67 antibody (20-22). The M, 86,000 antigen has been shown to be associated with a M, 70,000 peptide and binds to DNA (19). The M, 86,000-70,000 proteins are very similar to the Ku antigen (M, 80,000) recognized by autoimmune sera (23, 24). The antigen recognized by the Ki 67 antibody is present in S and G2 phases and remains bound to the mitotic chromosomes. The Ki 67 antibody has been proposed as a prognostic tool in evaluating the proliferative degree of malignant non-Hodgkin's lymphoma (21), as has a nucleolar PCNA recognized by a polyclonal serum (9). A M, 145,000 nucleolar antigen has been identified (25) which is associated with growing cells (26). The M, 145,000 nucleolar protein was readily detectable in growing and dividing HL-60 human promyelocytic leukemia cells but not after retinoic acid-induced differentiation (27).

The present study was developed to identify potential PCNA proteins using monoclonal antibodies developed against HeLa nucleolar extracts. Several hybridoma clones from different fusions produced bright nuclear immunofluorescence on HeLa cells. These MAbs identified two novel nuclear antigens that were expressed specifically in cycling cells.

MATERIALS AND METHODS

Cells. Human tumor cell lines HeLa S3 and Hep-2 (obtained from the American Type Culture Collection, Rockville, MD), HL-60 (human promyelocytic leukemia, obtained from B. Dowell), human colon tumor lines HCT 116, GEO, JVC, and Moser (obtained from M. Brattain) and rat Novikoff cells (obtained from Bob Ochs) were grown in Dulbecco's minimum essential medium containing pyruvate, nonessential amino acids, and glutamine supplemented with 10% FCS. Hybridoma cells were grown in Dulbecco's minimum essential medium supplemented with 20% FCS. Cells were maintained at 37°C in a humidified 5% CO2 incubator.

Isolation of Nuclear Proteins. Nuclei were isolated from HeLa S3 cells as described by Yaneva et al. (19). Nuclear proteins were extracted from the isolated nuclei in 10 mM Tris-HCl (pH 8.0), 0.2 mM PMSF, 1 μg/ml leupeptin, and 1 μg/ml aprotinin. The mixture was centrifuged at 12,000 × g for 15 min after a 2-h incubation at 4°C, and the supernatant was collected as the source of DNA-associated protein. The isolated nuclei in 10 mM Tris-HCl (pH 7.5), 20 mM dithiothreitol, 0.2% deoxycholate, 10 mM KCl, 0.5 mM MgCl2, 0.5 mM PMSF, and 1 μg/ml leupeptin and aprotinin. The extracted nuclear proteins were partially purified from protein C23 by 30% (NH4)2SO4 precipitation, as described by Freeman et al. (25). The precipitated proteins were dissolved in 2 μl urea/1 μl NaCl and were used as the source of antigens for immunization.

Immunization. Female BALB/c mice were immunized with extracted nuclear proteins as described by Freeman et al. (29). Subsequent cell fusion and cloning were also as described by Freeman et al. (29). Initial screening of clones from several fusions revealed clones which reacted with whole nuclei and nuclear fractions of HeLa cells. Antibodies to
nuclear proteins could be the result of common nucleolar and nuclear elements or nuclear "contaminants" of the nucleolar extracts.

Antibody Typing. The class and subclass identity of each mouse monoclonal antibody was determined by Ouchterlony immunodiffusion using a typing kit obtained from Miles Scientific (ICN Immunobiologics, Irvine, CA). All four antibodies are IgGs. 5C2, 9F9, and 4F8 are monoclonal antibody was determined by Ouchterlony immunodiffusion and 5C7 is an IgG1; 5C7 is an IgG2β (Table 1).

Immunofluorescence. HeLa S3 cells were grown on 10-well microscope slides (Roboz Surgical). The cells were fixed with 2% paraformaldehyde in PBS, pH 7.4, for 30 min at room temperature and then washed 3 times with PBS for 15 min each. The fixed cells were permeabilized with acetone at −20°C for 10 min, followed by 3 washes in PBS. The cells were incubated with monoclonal antibody culture supernatants for 1 h at 37°C in a humidified chamber, followed by 3 washes for 15 min in PBS. The cells were incubated with a 1/150 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulins (Boehringer-Mannheim) at 37°C for 30 min, washed 3 times in PBS for 15 min each and observed with a Zeiss fluorescent microscope.

SDS-PAGE and Immunoblotting. Proteins were separated by electrophoresis on 7.5 or 12.5% SDS-polyacrylamide gels according to the procedure of Towbin (32). Proteins were either stained with 0.3% Coomassie brilliant blue or silver (31) or transferred to nitrocellulose paper (0.45 µm; Schleicher and Schuell, Keene, NH) as described by Towbin et al. (32). Immunoblotting of the bound proteins with the monoclonal antibodies was as described by Freeman et al. (29).

Protein Assay. Protein concentrations were determined by the Bio-Rad protein assay (33).

ELISA Assay. Antigen was absorbed onto microtiter plates (Immunon I) overnight at 4°C. Nonspecific binding was inhibited by blocking in 10% chicken serum-3% bovine serum albumin in PBS, pH 7.5, for 1 h. The monoclonal antibodies (hybriDNA culture supernatants) to nuclear proteins were the primary antibodies, and goat anti-mouse conjugated to peroxidase (Jackson Immunoresearch) diluted 1:1000 in PBS:0.5% Tween 20 was the secondary antibody. The color was developed by the addition of 0.02% H2O2 and 300 nM 2,2′-azino-di-[3'-ethylbenzthiazolin-sulfonate (6)]. The reaction was terminated after 30 min by the addition of 1 M NaF. The absorbance was read at 405 nm on an ELISA reader (Fischer).

Sucrose Gradient Fractionation. HeLa cell nuclear extracts in 10 mM Tris·HCl, pH 8.0, with protease inhibitors (1 µg/ml leupeptin, aprotinin, and 0.2 mM PMSF) were layered on top of linear 5–45% (w/v) sucrose density gradients, made up in the same buffer. The gradients were centrifuged for 16 h at 28,000 rpm on an SW28 rotor (Beckman, Munich, Germany) at 4°C. Gradient fractions of 0.4 or 1.2 ml were collected, and antigen was detected by ELISA assay and immunoblotting. Lactate dehydrogenase (7.3S), catalase (11.3S), and apoferritin (17.6S) were used as markers.

Cell Cycle Analysis. Normal human peripheral blood lymphocytes (obtained from four individuals at separate times) were isolated from 30 ml of peripheral blood using Ficoll type FP (Sigma; F8626). The isolated lymphocytes were counted and 1 x 10⁶ cells/ml were added to RPMI 1640 (K. C. Biological) supplemented with 10% FCS (Hazelton). In PHA-stimulated cultures, the concentration of PHA was 5 µg/ml (Sigma; L-8504); control cultures did not contain PHA. The cells were incubated for 72 h at 37°C in a humidified 5% CO2 incubator.

For cell cycle analysis, isolated lymphocytes were cultured as described above except autologous human serum was used instead of FCS. Control cells were taken at 0 h, prior to addition of PHA. Cells were collected after PHA stimulation at 6, 22, 44, and 68 h. These studies were done in two independent experiments.

Cells were attached to a slide surface by cytocentrifugation at 1000 rpm for 5 min. The cells were prepared for immunofluorescence as described above.

RESULTS

Immunofluorescence Localization. Those antigens exhibiting nuclear localization in HeLa cells were tested for PCNA-like properties by comparative immunofluorescence in resting and PHA-stimulated lymphocytes and in tumor cell lines. Four MABs (5C2, 9F9, 4F8, and 5C7) were selected for further study because three of these antigens possessed PCNA-like properties (5C2, 9F9, and 4F8) and one antigen (5C7) was present in all cells and was used in these studies as a control. MABs 5C2 (Fig. 1D) and 9F9 (Fig. 1B) which recognized a M, 110,000 protein (see below) and MAB 4F8 (Fig. 1C) which recognized a M, 85,000 protein each produced bright nuclear immunofluorescent staining in HeLa S3 cells; in each case the nucleoli were negative and appeared as dark spots. With these MABs, the immunofluorescent staining at mitosis was found throughout the cell and was not associated with the chromosomes. On the other hand, MAB 5C7 which recognized a M, 18,000 antigen produced bright homogeneous nuclear immunofluorescent staining in HeLa S3 cells and the nucleoli were included in the immunostaining. At mitosis, the immunofluorescence remained associated with the condensed chromosomes. Other human tumor cell lines and Novikoff hepatoma cells had the same immunofluorescent staining pattern as seen in HeLa S3 for all four monoclonal antibodies (Table 1).

Immunoblot Analysis. Immunoblots prepared with these MABs using rabbit anti-mouse antibody as a second antibody and 125I-Protein A, were used to determine the molecular weights of the antigens. Nuclear extracts (Fig. 2, Lane A) from HeLa S3 cells were the source of antigen. MABS 5C2 and 9F9 (Fig. 2, Lanes B and C) recognized an approximately M, 85,000 polypeptide. Competitive immunoblots revealed that these two MABS recognize the same antigen (data not shown). A M, 85,000 polypeptide was recognized by MAB 4F8 (Fig. 2, Lane D); and MAB 5C7 (Fig. 2, Lane E) recognized a polypeptide with a molecular weight of approximately 18,000.

Sucrose Density Gradient Analysis. HeLa S3 nuclei extracted in 0.01 M Tris, pH 8.0, were subjected to linear sucrose density gradient fractionation to determine whether the polypeptides recognized by the individual antibodies were associated with any high molecular weight particles (deoxyribonucleoprotein or ribonucleoprotein). Peak ELISA activity of MAB 5C2 and 9F9 (p110) was found in fraction 20 (Fig. 3, a and b, respectively) which corresponds to a particle of 10–15S. Analysis for MAB 4F8 (p85) showed immunoreactivity in the ELISA assay in fraction 16 (Fig. 3c), which corresponds to an approximately 10S particle.

Every fourth fraction was applied to a 7.5% SDS gel, and the proteins were separated by electrophoresis. Silver staining revealed that most fractions contained many proteins (Fig. 4) when compared with the original nuclear extract (Fig. 4J). Fractions 12, 16, and 20 (Fig. 4, Lanes G, F, and E respectively) contained protein bands which corresponded to the M, 110,000

Table 1 Comparison of the four nuclear monoclonal antibodies

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<tr>
<th>Antigen molecular weight</th>
<th>5C2</th>
<th>9F9</th>
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+++. low level positive immunofluorescence; ++, high positive immunofluorescence; ++++, very intense immunofluorescence.
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Fig. 1. Immunofluorescence of HeLa cells fixed in 2% paraformaldehyde and permeabilized with acetone. (A) MAb 5C2 labels the nuclei of interphase cells but does not label the nucleoli. In mitotic cells, the label is in the cytoplasm and is not bound to the chromosomes, as seen here in an anaphase cell (arrow). (B) MAb 9F9 labels the interphase nuclei but is not found in the nucleoli. As with MAb 5C2 and 9F9, mitotic chromosomes were not stained with MAb 4F8. (C) MAb 5C7 has a homogeneous nuclear staining pattern and is found associated with the chromosomes at mitosis (arrows). (E) Hybridoma-negative control shows background staining. (F) Phase-contrast microscopy of E.

and the M, 85,000 proteins. When gels were transferred to nitrocellulose and immunoblotted, the M, 110,000 protein was mainly detected in fractions 16 and 20 (Fig. 5A) and the M, 85,000 protein was detected in fractions 16 and 20 (Fig. 5B). The immunoblot data on p110 and p85 confirmed the results obtained by ELISA on sucrose density gradient fractionation.

When the ELISA activity of MAb 5C7 (p18) was tested with 0.01 M Tris nuclear extracts subjected to sucrose density gradient fractionation, no definitive peak was found (Fig. 6A); instead, a gradual increase was found across the gradient. Immunoblot analysis of the gradient fractions with MAb 5C7 did not show detectable levels of p18.

When HeLa S3 nuclear extracts were treated with DNase I (Cooper Biomedical; 50 μg/ml/120 units) in the presence of 1.5 mM MgCl₂, p18 was released and was found by ELISA assay at the top of the gradient (Fig. 6B). The p110 and p85 polypeptides were unaffected by DNase I.

PCNA. To evaluate whether the polypeptides were associated specifically with proliferating cells, immunofluorescent localization was done on resting and PHA-stimulated human peripheral blood lymphocytes. In control resting lymphocytes, MAb 5C7 (p18) immunostained the nuclei; the pattern was similar to that obtained for human cancer cell lines (Table 1). Proteins p110 and p85 were not observed in control, resting lymphocytes by immunofluorescence. All four antibodies labeled PHA-stimulated lymphocytes in a pattern consistent with that obtained in HeLa S3 cells (Table 1).

Cell Cycle Analysis. To determine the time of expression of the antigens, a time course study was performed on PHA-stimulated normal human lymphocytes. The M, 18,000 polypeptide was detectable throughout the time course studied. The M, 85,000 protein was undetectable at 0 h; it was detected at 6 h and remained so throughout the time course. The M, 110,000 protein was undetectable at 0 and 6 h; it was weakly detectable at 22 and 44 h and was more evident at 68 h. Cells (1 × 10⁵) from each time point were dissolved in Laemmli buffer, separated by SDS-PAGE (Fig. 7A), and transferred to nitrocellulose. Immunoblot analysis detected the M, 110,000 protein at 68 h (Fig. 7B, Panel a, Lane E); the M, 85,000 protein was detected at 44 and 68 h (Fig. 7B, Panel b, Lanes D and E); and the M, 18,000 protein was detected at 22, 44, and 68 h (Fig. 7B, Panel c, Lanes C, D, and E).

DNA Polymerase α₂ Complex. To determine whether the growth-related polypeptides (i.e., p110 or p85) were associated with the DNA polymerase α₂ complex of HeLa cells, purified DNA polymerase α₂, obtained from Earl F. Baril, Worcester Foundation, was fractionated on a 7.5% polyacrylamide gel and transferred to nitrocellulose. When reacted with the antibodies, p85 was not detectable in the fractionated complex. However, p110 appears as a prominent band in the fractionated complex (Fig. 8).

DISCUSSION

Of the proteins detected with the four monoclonal antibodies, the M, 18,000 peptide was located exclusively in the nuclei of
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Fig. 2. SDS-PAGE and immunoblotting. Proteins extracted from HeLa nuclei were fractionated by 7.5% (Lanes A–D; molecular weight markers on left) or 12.5% (Lane E; molecular weight markers on right) SDS-PAGE, transferred to nitrocellulose and immunoblotted. (A) Coomassie blue stain gel of nuclear extract. (B) A M, 110,000 protein was immunostained with MAb 522. (C) A similar M, 110,000 protein was immunostained with MAb 9F9. (D) A M, 85,000 protein was immunostained with MAb 4F8. (E) A M, 18,000 protein was immunostained with MAb 5C7.

Fig. 3. ELISA analysis of sucrose density gradient fractions. Nuclear extracts were applied to a 5–45% (w/v) linear sucrose density gradient and centrifuged at 28,000 rpm for 16 h. After fractionation, ELISA activity and protein concentration were determined for each fraction. (a) Peak ELISA activity of MAb 5C2 (p110) occurs in fractions 20–22 which corresponds to the second protein peak. (b) Peak ELISA activity of MAb 9F9 (p110) also occurs in fractions 20–22. (c) Peak ELISA activity for MAb 4F8 occurs in fraction 16, protein concentration; O, ELISA activity; , absorbance at 254 nm; Fraction 0, top of gradient; Fraction 60, bottom of gradient.

Fig. 4. Silver-stained SDS-PAGE of sucrose density gradient fractions; 100 µl of each fraction or 50 µg of original 0.01 M Tris nuclear extract were diluted in 2x Laemmli buffer and applied to a 7.5% gel. Lane A, fraction 36; Lane B, fraction 32; Lane C, fraction 28; Lane D, fraction 24; Lane E, fraction 20; Lane F, fraction 16; Lane G, fraction 12; Lane H, fraction 8; Lane I, fraction 4; Lane J, original extract. Arrowhead, position of p110; arrow, position of p85.

At mitosis, p18 is associated with the condensed chromosomes in all cell lines examined. The p18 peptide was found at the top of a linear sucrose density gradient following DNase I treatment of the nuclear extract, suggesting that it is probably bound to the DNA. Immunoprecipitation experiments using 32P in vivo labeled DNA confirmed that p18 binds DNA (data not shown). The M, 18,000 protein was present in normal resting lymphocytes as well as PHA-stimulated lymphocytes.

The presence of p18 on mitotic chromosomes and its presence in noncycling cells suggests that it may be a structural protein of the nucleus and/or chromosomes. The M, 18,000 antibody cross-reacts with Novikoff hepatoma and PTK2 cells, as well as human cells. The antibody does not recognize any of the histones based on immunoblot analysis of purified histones (data not shown). Based on molecular weight, p18 is too large to be ubiquitin (M, 8500), HMG proteins HMG-17 (M, 9000) or HMG-24 (M, 12,000). Therefore, p18 is probably a novel nuclear DNA-associated protein which may be a potentially useful tool for studies on nuclear and chromosome structure and function.

The M, 85,000 nuclear protein was localized exclusively in the nuclei and was not found in the nucleoli. At mitosis, the protein migrated to the cytoplasm and did not associate with the chromosomes. The M, 85,000 protein was found in all tumor cell lines examined, but was not present in normal resting (G0) lymphocytes. Upon PHA stimulation, p85 appeared within 6 h after induction and was present thereafter at all time points examined. The appearance of p85 within 6 h following PHA stimulation of lymphocytes may correlate with initiation of RNA synthesis (34). Although p85 may have a role in RNA synthesis, further studies on this point are required.

Although p85 has PCNA-like properties, it is not the M, 86,000 PCNA described by Zweig et al. (12) and Yaneva et al. (19); its immunofluorescence distribution differs and on immunoblot analysis this MAb did not react with purified M,
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Fig. 5. Immunoblot analysis of gradient fractions shown in Fig. 4 for detection of p110 (arrowhead). Most activity is seen in fractions 16 and 20. (B) Immunoblot analysis of gradient fractions shown in Fig. 4 for detection of p85. p85 (arrowhead) is detected in fractions 16-24, with minor amounts detected in other fractions.

Fig. 6. Sucrose density gradient analysis of MAb 5C7 (p18) localization. (A) Sucrose density gradient fractions of 0.01 M Tris nuclear extracts were assayed for ELISA activity. No definitive peak was observable. Instead, the ELISA activity appeared to increase across the gradient. (B) Sucrose density gradient fractionation of DNase I-treated nuclear extracts released p18 to the top of the gradient, as determined by ELISA assay. O, ELISA activity; A, protein concentration; ——, absorbance at 254 nm; S markers are indicated by arrows.

Fig. 7. (A) Coomassie-stained 7.5% polyacrylamide gel of control and PHA-stimulated normal human lymphocytes. Cells (1 x 10⁶) from each time point were solubilized in Laemmli buffer and fractionated by SDS-PAGE. Lane A, 0 h control; Lane B, 6 h following PHA stimulation; Lane C, 22 h following PHA stimulation; Lane D, 44 h following PHA stimulation; Lane E, 68 h following PHA stimulation. The large bands seen at M, 50,000 (arrowheads) are the heavy chains of IgG contained in the samples. (B) Immunoblot analysis of control and PHA-stimulated lymphocytes shown in A. p85 appears following 44 h of PHA stimulation (arrowhead, Panel a). p85 appears following 44 h of PHA stimulation (arrowhead, Panel b). Immunoblot analysis of p18 (on 12.5% gel). In Panel c, p18 appears at 22 h following PHA stimulation (arrowhead). Lane A, 0 h; Lane B, 6 h; Lane C, 22 h; Lane D, 44 h; Lane E, 68 h. The decreased immunoreactivity seen in those immunoblots is due to the reduced amount of specific protein available. The nuclei were not isolated from the lymphocytes; instead, whole cells were used.

The p110 protein is localized to the nuclei of cycling cells and is not found in the nucleoli based on immunofluorescent studies. At mitosis, the protein is distributed throughout the cell. It is not associated with the mitotic chromosomes. The M₉, 110,000 protein was found in all tumor cell lines investigated.
may be in the prekinetochores (47). In human mitotic cells, topoisomerase I is localized over the chromosomes (47). The $M$, 110,000 antigen detected in these studies differs from the previously reported $M$, 110,000 antigens.

A Ki 67 monoclonal antibody also recognizes a nuclear PCNA but the molecular weight of the antigen has not been reported (20–22, 48). Since the Ki 67 antibody remains associated with the chromosomes during mitosis (20), it is unlikely that the PCNA-like antigens described here are related to the antigen recognized by Ki 67. Only the $M$, 18,000 antigen remains associated with the chromosomes, and it does not have PCNA-like properties.

These data show that both p110 and p85 are PCNA-like proteins. The p85 protein appears in G1 (6 h after PHA stimulation), and p110 appears following 22 h of PHA stimulation in S phase. Protein p18 is not PCNA-like and may be a structural protein.

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CELL CYCLE-RELATED NUCLEAR PROTEINS FOUND WITH Mabs


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