Identification and Partial Characterization of a M, 105,000 Nucleolar Antigen Associated with Cell Proliferation

Amitava Chatterjee, James W. Freeman, and Harris Busch

Baylor College of Medicine, Department of Pharmacology, Houston, Texas 77030

ABSTRACT

This study reports the identification and partial characterization of a novel M, 105,000 nucleolar antigen (P105) identified by a monoclonal antibody. This monoclonal antibody was obtained when a nucleolar protein extract separated from the immunodominant protein C23 was used as the immunogen. Nucleolar antigen P105 was not detected in normal (resting) human liver, kidney, or peripheral blood lymphocytes but was present in a variety of human malignant tissues and cells. Lymphocyte nucleoli also exhibited specific P105 staining after 72 h of phytohemagglutinin stimulation. Nucleolar antigen P105 was detected in growing and dividing HL 60 cells but was not detected in retinoic acid-induced differentiated HL 60 cells. When HeLa cells were made quiescent by 48 h of serum starvation, the P105 antigen was not detected, but after refeeding with serum-containing medium, the antigen P105 was detected in the HeLa nucleoli within 2 h. These results indicate that nucleolar antigen P105 is a proliferating cell nuclear and nucleolar antigen-like molecule which appears early in the G1-S phase of the cell cycle.

INTRODUCTION

Pleomorphism of nucleoli is commonly found in malignant tumor cells (1). Nucleolar proteins are of special interest because of their potential roles in synthesis, processing, packaging, and transport of ribosomal RNA (2-4). Qualitative and quantitative differences of nucleolar proteins in tumor cells and normal tissues have been reported in both biochemical (5-10) and immunological (11-22) studies. PCNA-like antigens were previously reported (9, 10, 13-15, 17). The PCNA-like proteins are preferentially synthesized in dividing cells and react with proliferating cells, including tumor cells, but are present in little or undetectable amounts in normal (resting) cells. In studies on nucleolar antigens absent from normal resting tissues but present in proliferating tissue recently Freeman et al. (23) reported a monoclonal antibody to a M, 145,000 nucleolar antigen, and Chatterjee et al. (24) described a monoclonal antibody to a M, 40,000 nucleolar antigen.

The present study reports the identification of a novel M, 105,000 nucleolar antigen by a monoclonal antibody. This newly identified nucleolar antigen P105 was not detected in normal liver, kidney, and lymphocytes but showed bright nucleolar immunofluorescence in a variety of human malignant tissues and cells. This newly identified nucleolar antigen P105 is different from cyclin, a PCNA protein (13) with a molecular weight of 35,000, whose rate of synthesis increased during S phase, at which time it translocates from the nucleoplasm to the nucleolus. This P105 nucleolar antigen has PCNA-like properties. It is absent from normal blood lymphocytes but appears in significant amounts in the nucleoli of PHA-activated lymphocytes after 72 h. In growing and dividing HL 60 cells, the antigen is present, but it is lost during differentiation. It is very weakly detectable in the nucleoli of serum-starved HeLa cells, but upon addition of fresh medium, P105 antigen appears in the nucleoli within 2 h. P105 which is absent from normal rat liver was found in nucleoli of regenerating rat liver. The M, 105,000 nucleolar antigen identified here is therefore associated with cell proliferation.

MATERIALS AND METHODS

Antigen Preparation and Production of Monoclonal Antibodies. Nucleoli were isolated from 10 g of HeLa cells as previously described (25). Nucleolar proteins were extracted from nucleoli by homogenization in 10 mM Tris-HCl (pH 8.0)/0.2% deoxycholate/20 mM dithiothreitol/10 mM KC1/0.5 mM MgCl2/0.5 mM phenylmethylsulfonyl fluoride/leupeptin (5 ¿g/ml)/aprotinin (10 ¿g/ml). After extraction, the mixture was centrifuged at 25,000 x g for 30 min. The supernatant containing extractable nucleolar proteins was made to 30% saturated ammonium sulfate, and the proteins were allowed to precipitate for 3 h at 4°C. The mixture was spun at 12,000 x g for 15 min. The pellet largely free from protein C23 was dissolved in 1 M NaCl/2 M urea and used for immunization. The methods for production of hybridomas and cloning were previously described (25).

Immunohistological Study. Immunocytochemical localization studies were done following the method of Freeman et al. (23).

Polyacrylamide Gel Electrophoresis. Electrophoretic analysis of proteins was performed on 7.5% polyacrylamide gels with 0.1% SDS according to the method of Takacs (26). Nucleolar proteins were dissolved in boiling Laemmli buffer (27) for 3 to 5 min. The sample was loaded on the gel and electrophoresed for 2.5 h at 40 mA.

Immunoprecipitation of Nucleolar Antigen P105. The HeLa nucleoli were extracted with 10 mM Tris-HCl (pH 8.0) containing 0.5 mm phenylmethylsulfonyl fluoride/leupeptin (5 ¿g/ml)/aprotinin (10 ¿g/ml). The mixture was then centrifuged at 15,000 rpm (27,000 x g) for 30 min. The clear supernatant was used immediately as the source of antigen. The proteins in the nucleolar Tris extract (200 liters) were radiolabeled (32P) by the chloramine-T method (23). For immunoprecipitation, 10 ¿l of anti-P105 (immunoglobulin) monoclonal antibody were added and allowed to react at room temperature for 2 h. Rabbit anti-mouse antibody (Cappel) was added (1 ¿g/ml), and the mixture was kept for 1 h at room temperature. Then, 250 ¿l of Pansorbin (Calbiochem) were added, and the mixture was incubated further for 30 min. The whole complex was then pelleted by centrifugation (12,000 x g) and washed 5 times with wash buffer [150 mm NaCl/5 mm EDTA/50 mm Tris/0.25% gelatin/0.05% Nonidet P-40 (pH 7.4)]. The washed pellet was dissolved in Laemmli's buffer and electrophoresed on a 7.5% gel. The gel was then dried and exposed to XAR-5 (Kodak) film to develop the autoradiogram.

Immunofluorescence Chromatography. Anti-P105 immunoglobulin was isolated from culture supernatant and was coupled to CNBr-activated Sepharose 4B (Pharmacia) (24). HeLa nucleolar proteins [30 mg in 10 mM Tris-HCl (pH 8.0)] were mixed with the anti-P105 MAb-coupled Sepharose 4B and allowed to react overnight at 4°C by tumbling end over end. The unbound proteins were eluted with 0.1 M phosphate buffer containing 0.5 mm NaCl (pH 7.4), and the bound antigen was eluted with 4 M MgCl2 (24). The sample was dialyzed overnight against...
1000 volumes of 0.01 \text{m Tris-HCl} (\text{pH} 8.0) and used for ELISA and gel electrophoresis.

Peptide Mapping. The immunoaffinity-purified P105 antigen was run on a 7.5\% SDS-polyacrylamide gel, and the Coomassie blue-stained band was cut out from the gel. The protein in the gel slice was labeled with \(^{125}\text{I}\) by the chloramine-T method (23). The gel slice was washed with 10\% methanol for 2 to 3 days with several changes and then was digested with trypsin (50 \text{m\&g/m\&l})/0.5 \text{m NaHCO}_3 \text{ buffer (pH 8.0)} at 37\text{\degree C} for 20 h. The supernatant was collected, lyophilized, and analyzed on cellulose-coated thin-layer chromatography plates. Electrophoresis was carried out at pH 1.9 (acetic acid/formic acid/water, 15:5:80) on a Savant high voltage electrophoresis apparatus at 400 V for 2 h. The peptides were run in the second dimension in butanol/pyridine:acetic acid/water (32.5:25:5:20) for chromatography. The dried plate was exposed to XAR-5 Kodak film at -70\text{\degree C}.

Retinoic Acid-induced Differentiation of HL 60 Cells. HL 60 cells (human promyelocytic leukemia cell line) were grown in suspension cultures in RPMI 1640 medium (Grand Island Biological Co.) in a humidified atmosphere with 5\% CO\(_2\) containing 10\% fetal calf serum, 100 IU penicillin per ml, 100 \text{m\&g/m\&l} streptomycin per ml, and transferrin at a concentration of 1 \text{m\&g/ml/} (Sigma). The cells were treated with retinoic acid for 120 h. During the treatment, cells were collected at different time points and were attached to the slides by cyt centrifugation. A side-by-side control of untreated HL 60 cells was analyzed. The P105 nucleolar antigen was detected by indirect immunofluorescence. Antigen P105 was quantitated in control and retinoic acid-treated HL 60 cells using an ELISA assay.

Serum Deprivation Study. HeLa cells were grown for 48 h in DMEM containing 10\% fetal calf serum in a humidified atmosphere with 5\% CO\(_2\). After 48 h, the complete medium was washed out and replaced by a DMEM lacking fetal calf serum; the cells were grown for another 48 h. After 48 h of serum starvation, a sample was taken at 0 h, and the medium was replaced with fresh DMEM containing 10\% fetal calf serum. At 1-h intervals cells were collected and cyt centrifuged onto slides, and antigen P105 was detected by indirect immunofluorescence.

PHA Activation Study in Blood Lymphocytes. Using Ficoll-Hypaque (Sigma) gradients, human peripheral blood lymphocytes were isolated and cultured up to 96 h in RPMI 1640 medium (Gibco) containing 10\% fetal calf serum and 5 \text{g/ml PHA (Sigma). At 24, 48, and 72 h, cells were collected and analyzed for immunolocalization.}

ELISA. Immunoaffinity-purified P105 was added to polystyrene microtiter plates (Dynatech Immulon 1) and bound overnight at 4\text{\degree C}. Excess binding sites were then blocked with the blocking buffer containing 7\% chicken serum, 3\% bovine serum albumin, and 0.05\% Tween 20 in PBS. The wells were washed with PBS/0.05\% Tween 20 (pH 7.4); 50 \text{m\&l} of culture supernatant were added to the wells, and the plate was placed on a rotary shaker for 2 h. The wells were washed 3 times with PBS/0.05\% Tween 20, and peroxidase-conjugated rabbit anti-mouse immunoglobulin (Cappel) was added and incubated for an additional hour at room temperature. The wells were washed 5 times in the same buffer, and the substrate was added [2,2'-azinodi-3-ethyl benzthiazoline sulfonate in 0.1 \text{m sodium citrate (pH 4.0)} containing 0.5 \text{m\&l of 30\% hydrogen peroxide}]. After 15 to 30 min of incubation with shaking (ambient temperature), the enzyme reaction was stopped with the addition of NaF at 0.25 \text{m concentration}, and absorbance measurements made at 405 nm.

RESULTS

Expression of Nucleolar Antigen P105 in Various Tissues. The anti-M, 105,000 MAb identified a nucleolar protein present in growing and dividing cells that was absent from normal (resting) tissue (Tables 1 and 2). Fig. 1 compares immunocytochemical localization of P105 in normal liver (Fig. 1B) with tumor cells and tissue sections (Fig. 1, A, C to F). The anti-P105 monoclonal antibody produced bright nucleolar fluorescence in HeLa cells (Fig. 1A) and other types of tumor cells (Fig. 1, C and D) and tumor tissues (Fig. 1, E and F), but normal liver was negative for nucleolar fluorescence (Fig. 1B). In some regions (dual regions) of normal colon epithelium and hypertrophic prostate, nucleolar fluorescence was observed (Fig. 2, A and B). The nucleolar antigen P105 was undetectable in normal rat liver, but when rats were partially hepatectomized to study the induction of P105 in regenerating liver, nucleolar fluorescence was observed at 18 h after partial hepatectomy (Fig. 3, A and B). P105 was also found in nuclei of Novikoff hepatoma cell line (Fig. 3C).

Expression of P105 in PHA-activated Blood Lymphocytes. P105 was not detectable in normal (resting) blood lymphocytes (Fig. 4A). When the lymphocytes were stimulated by PHA, P105 was not observed at 24 h (Fig. 4B), but was present in nuclei and nucleoli at 48 h (Fig. 4C); it was more concentrated in nucleoli only after 72 h of PHA treatment (Fig. 4D).

Effect of Differentiation and Serum Starvation on the Expression of Nucleolar Antigen P105. HL 60 cells were used to study the expression of nucleolar antigen P105 in dividing and in terminally differentiated cells. For differentiation, HL 60 cells were treated with retinoic acid (1 \text{\mu M concentration}) for 120 h as previously described (28). At this point, approximately 95\% of the HL 60 cells had been found to be differentiated to metamyelocytes, banded, or segmented neutrophils (28). The anti-P105 MAb produced bright nucleolar fluorescence in cycling HL 60 cells (Fig. 5A), but the immunofluorescence decreased in 48- and 96-h retinoic acid-treated HL 60 cells (Fig. 5, B and C) and was not detectable at 120 h (Fig. 5D). Control and retinoic acid-treated HL 60 cells were extracted with 8 \text{m urea}, and the same amount of proteins was used to assay the P105 antigen by ELISA procedures. Fig. 6 shows that the P105 content was greater in proliferating cells (A) than in nonproliferating cells (B).

To further study the time point of P105 expression in the cell cycle, HeLa cells were arrested by serum deprivation for 48 h.
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Fig. 1. Immunocytochemical localization of P105 nucleolar antigen in HeLa cells (A), normal human liver (B), Hep 2 cells (C), acute myeloid leukemia (D), squamous cell carcinoma of the larynx (E), and lymphoma (F). Tissue sections or cells were fixed and permeabilized in acetone and reacted with the monoclonal antibody anti-P105. Following incubation with primary antibody, the antigen was detected using a fluorescein-conjugated rabbit anti-mouse second antibody.

Fig. 2. Immunocytochemical localization of nucleolar antigen P105 in normal colon (A) and hypertrophied prostate (B). The method used is described in the legend to Figure 1.

Bright nucleolar fluorescence was observed in fed cells (Fig. 7A), whereas very weak or undetectable nucleolar fluorescence was observed after 48 h of serum starvation (Fig. 7B). Immunofluorescence studies of serum-deprived HeLa cells refed with serum showed that the P105 antigen was detected after 2 h (Fig. 7C) of refeeding and reached its original intensity by 4 h (Fig. 7D). Interestingly, after 2 h of refeeding fluorescence was observed both in nucleoplasm (Fig. 7C) and nucleolus, whereas after 4 h of refeeding the antigen was present in the nucleolus only (Fig. 7D). This result shows that the P105 antigen appears early in the G1 phase in these cells.

Characterization of Nucleolar Antigen P105. Repeated experiments failed to give positive immunoblots using HeLa nucleolar extracts, suggesting that the P105 epitope was sensitive to this procedure. To identify the molecular weight of this nucleolar antigen, immunoprecipitation was performed. The immunoprecipitated antigen had a molecular weight of 105,000 (Fig. 8).

HeLa nucleolar protein P105 was also purified using an anti-P105 immunoaffinity column; the P105 protein was eluted with 4 M MgCl2, electrophoresed in a 7.5% SDS-polyacrylamide gel, and stained with Coomassie blue; and the major stained band was excised and used for peptide mapping. The tryptic map of P105 (Fig. 9, left) showed at least 18 spots and was not related to tryptic maps of other known nucleolar proteins such as P145 (23), C23 (Fig. 9, middle), or topoisomerase I (Fig. 9, right).

DISCUSSION

Recent studies (18, 23, 24) have shown significant qualitative and quantitative differences in nondividing and dividing cells. Some antigens which are PCNA-like are absent from normal (resting) cells. The M, 145,000 (23) and 40,000 (24) nucleolar antigens reported by this laboratory are examples of proteins associated with cell proliferation and were absent from nondividing cells or tissues. In this study, a novel M, 105,000 nucleolar antigen identified by a specific monoclonal antibody was shown to be another example of a PCNA-like antigen (13–15, 29). The anti-P105 MAb produced bright nucleolar fluorescence in HeLa and other human tumor cells and tissues, but not in normal human (resting) tissues. In growing nontumor tissues, nucleolar antigen P105 was detected in regions undergoing rapid growth, e.g., the ductal regions of hypertro-
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Fig. 3. Immunocytochemical localization of nucleolar antigen P105 in normal (resting) rat liver (A), regenerating rat liver (18 h) (B), and Novikoff cells (C). The method used is described in the legend to Fig. 1. The arrows indicate nucleolar fluorescence.

Fig. 4. PHA stimulation study with resting blood lymphocytes: control lymphocytes (A); 24 h after stimulation (B); 48 h after stimulation (C); and 72 h after stimulation (D). The experimental procedure was described in "Materials and Methods."

Fig. 5. Immunocytochemical localization of P105 antigen in HL60 cells (control (A), 48 h (B), 96 h (C), 120 h of retinoic acid treatment (D). The method used is described in "Materials and Methods."

Fig. 6. Quantitation of P105 nucleolar antigen by ELISA in proliferating HL60 cells (A) and retinoic acid-treated (120 h) HL60 cells (B). The experimental procedure was described in "Materials and Methods."

Like other PCNA molecules, nucleolar antigen P105 was not present in nucleoli of noncycling cells. In growing and dividing HL 60 cells, the anti-P105 MAb produced bright nucleolar fluorescence, but in the retinoic acid-induced differentiated HL 60 cells, P105 was not detected. Antigen P105 was not detected in growth-arrested HeLa cells after 48 h of serum deprivation. When the HeLa cells were refed with complete serum-containing medium, the P105 nucleolar protein was detected within 2 h. These results suggest that the P105 may be associated with cell cycle events and that it is synthesized early in the G1 phase in the nucleolus. Like P145 and P40 antigens, P105 is a PCNA protein with a specific nucleolar localization.

In ELISA, anti-P105 monoclonal antibody does not react with pure C23 or topoisomerase I. These results were also...
confirmed by tryptic peptide mapping which showed that P105 nucleolar antigen was not related to other known nucleolar proteins such as P145 (23), protein C23, or topoisomerase I. The M, 105,000 nucleolar protein is a unique and hitherto unreported nucleolar protein which may have value for detecting proliferating cells in tumor tissues.
REFERENCES


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