Expression of Human Proliferation-associated Nucleolar Antigen p120

Sissy M. Jhiang,¹ Mariana Yaneva, and Harris Busch²
Department of Pharmacology, Baylor College of Medicine, Houston, Texas 77030

Abstract
The amount of human nucleolar protein p120 is small or undetectable in most normal resting cells but increases greatly in some actively proliferating cells. The time course was compared for accumulation of mRNAs for p120, c-myc, and histones in phytohemagglutinin-stimulated peripheral blood mononuclear cells. The mRNAs for p120 and c-myc were undetectable in unstimulated peripheral blood mononuclear cells; phytohemagglutinin stimulation increased the p120 mRNA level within 2 h, and it reached a maximum within 24–48 h. The c-myc mRNA level increased within 4 h and had a biphasic maximum at 4 h and 24 h. Indirect immunofluorescence staining and Western blot analysis showed an increase in the level of p120 protein in the nucleolus following the increase in its mRNA level, which suggests that p120 expression is transcriptionally controlled. Both p120 mRNA and c-myc mRNA levels were significantly decreased in 12-O-tetradecanoylphorbol-13-acetate-differentiated HL-60 leukemic cells and in confluent normal immortalized human fibroblasts (W51). These data indicate that p120 is expressed very early in the G1 phase of the cell cycle and suggest that it has an important function in the cell cycle.

Introduction
In contrast to the small, spherical nucleoli of normal resting cells, the nucleoli of rapidly proliferating cancer cells are pleomorphic and hyperactive. Isolation and characterization of proliferating cell nucleolar antigens have been a major effort of our laboratory (1–4). The p120 nucleolar antigen (4) was found in a broad range of human tumors but was undetectable or found in very low levels in most normal resting tissues by indirect immunofluorescence. It was also found in undifferentiated HL-60 cells and PHA³-stimulated PBMC. It was difficult to detect in nucleoli of 48-h serum-starved HeLa cells, retinoic acid-treated HL-60 cells, and unstimulated PBMC (4).

The p120 antigen is localized to a network of beaded fibrils distributed throughout the nucleolus (5). Since in situ nuclease digestion of cells did not release p120 from the nucleolus, the p120 protein may be a component of a nucleolar network that appears in the G1 phase of the cell cycle.

The cDNA clone for p120 was isolated by screening a λgt11 human fetal liver cDNA library with a monoclonal antibody to p120 (6). A genomic DNA clone was isolated from a human chronic myelogenous leukemia genomic library using the cDNA probe (7). The nucleotide sequences and the gene structure for p120 were determined (6, 7). Comparison with the sequences of other genes available in the Gene Bank showed that p120 was a novel protein.

In this study, the level of mRNA for p120 was determined in two systems, normal human lymphocytes stimulated to proliferate with lecin PHA and HL-60 leukemic cells induced to differentiate by the phorbol ester TPA. Specific cDNA probes were used to study the expression of p120 during cell proliferation.

Results
Immunofluorescence in PHA-stimulated Lymphocytes
Isolated lymphocytes were treated in culture with PHA and examined by immunofluorescent staining at different times of p120 protein expression (Fig. 1). Nucleolar antigen p120 was faintly detected in fresh PBMC (Fig. 1a) and in untreated lymphocytes maintained in culture for 96 h (Fig. 1k) with the 100X objective under oil. The immunofluorescence for p120 increased with time and reached maximum intensity in 40–48-h PHA-treated lymphocytes in parallel with increased nucleolar size. The doubling time of the mitogen-stimulated lymphocytes was about 96 h (8).

Immunoblot Analysis of p120 in PHA-stimulated Lymphocytes
To determine the accumulation of p120 during the time course of PHA stimulation of lymphocytes, equal numbers of cells were collected, dissolved in Laemmli sample buffer, and applied to SDS-polyacrylamide gels. The proteins were then transferred to nitrocellulose and reacted with the p120 monoclonal antibody (Fig. 2, A and B). The p120 protein was not detectable in fresh PBMC (Fig. 2, A and B) and untreated lymphocytes (Fig. 2B), but the p120 protein was shown to be significantly increased after 40 h of PHA stimulation (Fig. 2A). The HeLa and HL-60 cells contained more p120 protein per cell than the stimulated lymphocytes (Fig. 2B). The expression of p120 is apparently coupled with the proliferation activity of cells.

RNA Blot Analysis of PHA-stimulated Lymphocytes
Total cellular RNA was isolated from cultured PHA-treated PBMC at various times. Standard RNA blotting techniques were used to determine the time course of expression of p120, c-myc, and histone H2B. Protein p120 mRNA was detectable at 2 h and increased to a maximum at 24 h (Fig. 3). Fig. 3, A and B, represents the
Fig. 1. Time course of p120 immunofluorescence staining in PHA-stimulated PBMC. At various times of stimulation, cells were fixed, and the p120 protein was detected by indirect immunofluorescence. a and b, immunofluorescence and phase-contrast image of fresh unstimulated PBMC; c and d, 2 h after PHA stimulation; e and f, 24 h after PHA; g and h, 40 h after PHA; i and j, 72 h after PHA; k and l, cell cultured for 96 h without PHA. X 100.
results from two different experiments with different time points. The level of histone H2B mRNA increased at 48 h of stimulation, which marks the time of S-phase; histone mRNA increases during S-phase, when histone synthesis occurs (9). Experiments with [3H]thymidine incorporation have demonstrated that, at 48 h of stimulation, these cells enter S-phase (10, 11). A biphasic response of c-myc expression was observed as reported by Reed et al. (12). The level of c-myc mRNA increased within 4 h (Fig. 3A), and a second maximum at 24 h of stimulation was observed (Fig. 3, A and B).

RNA Blot Analysis of TPA-differentiated HL-60 Cells. As an alternate approach to that of mitogenic stimulation of lymphocytes, termination of cell proliferation was studied in TPA-differentiated HL-60 cells (13). As shown in Fig. 4, both p120 mRNA and c-myc mRNA levels significantly decreased in TPA-differentiated HL-60 cells.

Confluent normal immortalized human fibroblast WSI had a very low level of p120 mRNA and undetectable c-myc mRNA (Fig. 4). By indirect immunofluorescent staining, the level of p120 protein slightly declined in 24-h TPA-treated HL-60 and confluent WSI cells (data not shown). The difference between the mRNA and protein levels of p120 suggests a relatively long half-life of p120 protein in HL-60 cells.

Fig. 2. Immunoblot analysis of p120 protein during PHA stimulation of human lymphocytes. A, time course of p120 protein accumulation in PHA-stimulated PBMC; B, p120 protein accumulation in proliferating and nonproliferating cells. At various times of stimulation (indicated in h, above each lane), cells were counted, pelleted, and dissolved in Laemmli buffer. Cell extracts corresponding to equal numbers of cells were applied into SDS-polyacrylamide gel. The proteins were then transferred and reacted with p120 monoclonal antibody as described in "Materials and Methods." HeLa and HL-60 cells were included as positive controls.

Half-Life Estimates of P120 Protein and Transcripts in HL-60 Cells. The stabilities of p120 protein and message were assessed using cycloheximide to block protein synthesis and using actinomycin D to block RNA synthesis. The apparent half-life of the p120 protein in HL-60 cells was approximately 24 h (Fig. 5A), whereas the apparent half-life for the mRNA was about 60 min (Fig. 5B).
protein p120 was estimated to be about 24 h. The increased p120 mRNA level during PHA stimulation of PBMC indicated that expression of p120 mRNA was related to cell growth and proliferation. The decline of the p120 mRNA level in TPA-differentiated HL-60 cells suggests that expression of p120 mRNA is associated with cell proliferation and is decreased during differentiation as cell cycling is reduced. The p120 protein is involved in the proliferation because: (a) the amount of p120 protein and its mRNA are very low in G0 cells; both are greater in cycling cells; (b) the p120 protein is synthesized in early G1. Jaehning et al. (11) have shown that RNA polymerase I activity was continuously increased during 4 days of PHA stimulation in human lymphocytes, which parallels enlargement of the nucleolus as well as the amount of the p120 protein in the nucleolus. The increase of total cellular RNA during G1 phase of the cell cycle is mostly rRNA, and inhibition of rRNA synthesis by low doses of actinomycin D precludes DNA replication in stimulated lymphocytes (8). These results suggest that the p120 protein may have a role in increased rRNA synthesis during G1 phase of the cell cycle. The timing of appearance and the amounts of gene products are controlled by diverse mechanisms of synthesis and degradation. The 24-h apparent half-life of p120 protein in HL-60 cells (Fig. 5A) may or may not be the same as in nonproliferating cells. Further analysis is needed to determine whether cycloheximide may decrease protein(s) that degrade p120 protein, which stabilizes the p120 protein. In addition, immunoblot analysis is useful for quantitation only when the proteins detected are in the linear range but not in the saturation range.

Fig. 3. Time course of p120 mRNA level in PHA-stimulated PBMC. At various times indicated in hr., above each lane PBMC were collected from cultures; total cellular RNA was isolated and analyzed for relative levels of various mRNAs by Northern blot assay. Probe specificities are given at right. A and B represent results from two different experiments with different time points analyzed as indicated above the lanes. The blots were exposed 72 h for p120 and 22 h for c-myc and histone H2B.

Fig. 4. Changes in p120 mRNA level in HL-60 cells induced to differentiate by TPA. HL-60 cells treated with TPA dissolved in DMSO and HL-60 cells with or without DMSO were collected. Total cellular RNA was isolated and analyzed by Northern blot hybridization. RNA from HeLa cells and confluent W5 cells were analyzed as well. Probe specificities are given at right. The blots were exposed 72 h for p120 and 22 h for c-myc.

Discussion

This study shows that both mRNA and protein levels of p120 increased during PHA stimulation of human lymphocytes. The level of p120 mRNA was significantly increased as early as 2 h after stimulation. The maximal level of p120 mRNA was detected within 24-48 h, which followed the maximum of c-myc expression. The maximum for p120 mRNA occurred prior to the onset of DNA synthesis as determined by histone expression (8, 9). This result suggests that p120 may be one of the G1 genes that plays a role in commitment of cells to enter S-phase. The prompt induction of p120 mRNA within 2 h suggests that the p120 gene is regulated directly by the biochemical events that immediately follow mitogen binding in lymphocytes (14, 15).

The p120 mRNA level decreased in TPA-differentiated HL-60 cells. The apparent half-life for the mRNA was about 1 h in HL-60 cells. The apparent half-life of the
Polyclonal antisera to the p120 protein to quantitatively immunoprecipitate p120 protein will aid in determining its half-life.

The high level of expression of the p120 gene in HeLa cells may not be explained by gene amplification or gene rearrangements. Titration of the gene copy number by slot blots and genomic DNA Southern blots showed that there were no differences between human lymphocytes and HeLa cells (data not shown). The elevated level of p120 mRNA in proliferating cells may reflect the increased transcription rates or increased mRNA stability. The apparent half-life of p120 transcripts in HL-60 cells is approximately 60 min. The high steady-state level of p120 mRNA does not seem to be related to specific stabilization of the p120 message. Transcriptional control in p120 expression is most likely since induction of p120 protein by PHA is preceded by an increased expression of its mRNA. However, translational and/or posttranslational controls are also possible.

The molecular signals directly responsible for changes in p120 mRNA levels are not known. PHA indirectly increases protein kinase C activity in T-cells by stimulating calcium influx (16), and protein kinase C activity has been implicated in the induction of differentiation of HL-60 cells by TPA (17). It is possible that protein kinase C could be involved in regulation of p120 mRNA in proliferating cells; this would be important if the trans-acting factor(s) of p120 were phosphorylated.

Materials and Methods

Cells. Fresh buffy coats were obtained from the Gulf Coast Regional Blood Center. PBMC were isolated on a Histopaque-1077 gradient (Sigma). The cells were cultured at 2 × 10^6/ml in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum penicillin-streptomycin-PHA (10 μg/ml) (Sigma).

The HL-60 human promyelocytic leukemia cells were cultured at 6 × 10^7/ml in RPMI 1640 medium containing 10% fetal bovine serum penicillin-streptomycin-TPA (50 ng/ml) for 24 h. Untreated HL-60 cells were incubated side by side at the same time with or without the DMSO solvent for TPA (1:2000).

Wild human fetal skin fibroblasts were cultured in minimum essential medium with nonessential amino acids and 10% fetal bovine serum.

Indirect Immunofluorescence. Cells removed at different incubation times were attached to slides by cytocentrifugation for 5 min at 500 rpm (Cytospin 2; Shandon). The cells were fixed for 20 min with 2% paraformaldehyde (methanol free) in PBS and then washed three times with PBS for 5 min each time. The cells were then permeabilized in cold acetone at −20°C for 3 min and washed twice with PBS for 5 min; the slides were air dried. The primary p120 antibody was used at a dilution of 1:100 (ascites:PBS). The slides were incubated in a moist chamber at 37°C for 1 h. The mouse p120 antibody was detected with fluorescein isothiocyanate-conjugated goat anti-mouse immunoglobulin (Cappel) diluted 1:20 in PBS.

Polyacrylamide Gel Electrophoresis and Immunoblotting. The cells were pelleted, dissolved in Laemmli sample buffer, and heated in a 100°C heating block for 7 min. The dissolved samples were loaded on a 7.5% polyacrylamide gel with 0.1% SDS and electrophoresed for 1 h at 100 V. The proteins on the gel were transferred to a nitrocellulose membrane according to the manufacturer's manual (Bio-Rad Mini Trans-blot). Nonspecific binding sites on the membrane were blocked with 10% bovine serum albumin in TBST (10 mM Tris-HCl, pH 8.0–100 mM NaCl-0.05% Tween 20). The filters were incubated with the p120 antibody for 1 h at 1:200 dilution followed by three washings with TBST for 5 min each. The p120 antibody was detected with alkaline phosphatase-conjugated anti-mouse antibody (Promega).

RNA Isolation and Northern Blots. Total cellular RNA was prepared by a guanidium isothiocyanate procedure (18) and CsCl gradient centrifugation (19). RNA was quantitated by A_{260}, and 20 μg of total cellular RNA were fractionated on 1% agarose gels containing 1% formaldehyde-10 mM NaPO_4, pH 7.4. The RNA was transferred to Nytran membranes (Schleicher & Schuell) according to Thomas (20). The prehybridization and hybridization were performed as described by Schleicher & Schuell. Hybridization probes were labeled by nick translation with [1^2P]dCTP. The blots were finally washed in 0.1X saline-sodium phosphate-EDTA buffer-0.1% SDS at 65°C for 1 h. After each hybridization, RNA blots were stripped off the 3^2P-labeled probe by incubation at 65°C for 2 h in 5 mM Tris, pH 8.0–0.2 mM EDTA-0.05% pyro-
phosphate-0.1× Denhardt’s reagent. Blots were used three to four times.

Probes. The H2B and c-myc DNA probes were purchased from Oncor Probes. Histone H2B DNA was a chicken H2B cDNA clone. The c-myc DNA probe was a fragment released from a human genomic clone by ClaI/EcoRI digestion. The p120 DNA probe was derived from the cDNA obtained by Fonagy et al. (6).

Half-Life Studies. HL-60 cells were incubated at 37°C in the presence of cycloheximide (100 μg/ml) for various lengths of time. Whole HL-60 cell extracts were then subjected to polyacrylamide gel electrophoresis and immunoblotting as described above.

HL-60 cells were treated with actinomycin D (5 μg/ml) at 37°C for various periods of time and collected by centrifugation. Total cellular RNA was isolated and analyzed as described above.

References