

Up-regulation of Nucleolin mRNA and Protein in Peripheral Blood Mononuclear Cells by Extracellular-regulated Kinase*

Cara J. Westmark and James S. Malter‡

From the Institute on Aging and Department of Pathology and Laboratory Medicine, University of Wisconsin Medical School, Madison, Wisconsin 53792

Received for publication, October 16, 2000

Published, JBC Papers in Press, October 20, 2000, DOI 10.1074/jbc.M009435200

The signal transduction pathways regulating nucleolin mRNA and protein production have yet to be elucidated. Peripheral blood mononuclear cells treated with phorbol 12-myristate 13-acetate showed steady state levels of nucleolin mRNA that were 2–2.5-fold greater than untreated control cells. The up-regulation of nucleolin mRNA was substantially repressed by U0126, a specific inhibitor that blocks phosphorylation of extracellular-regulated kinase (ERK). Calcium ionophores A23187 and ionomycin also activated ERK and substantially elevated nucleolin mRNA levels, demonstrating phorbol 12-myristate 13-acetate and calcium signaling converge on ERK. Drugs that affected protein kinase C, protein kinase A, and phospholipase C signal transduction pathways did not alter nucleolin mRNA levels significantly. The half-life of nucleolin mRNA increased from 1.8 h in resting cells to 3.2 h with phorbol ester activation, suggesting ERK-mediated posttranscriptional regulation. Concomitantly, full-length nucleolin protein was increased. The higher levels of nucleolin protein were accompanied by increased binding of a 70-kDa nucleolin fragment to the 29-base instability element in the 3'-untranslated region of amyloid precursor protein (APP) mRNA in gel mobility shift assays. Supplementation of rabbit reticulocyte lysate with nucleolin decreased APP mRNA stability and protein production. These data suggest ERK up-regulates nucleolin posttranscriptionally thereby controlling APP production.

APP mRNA and protein levels is vital to understanding β -amyloid accumulation and deposition in Alzheimer's disease. Our laboratory has previously demonstrated that two RNA-binding proteins, nucleolin and heterogeneous nuclear ribonucleoprotein C (hnRNP C), bind to a 29-base instability element in the 3'-untranslated region (UTR) of APP mRNA (1). In rabbit reticulocyte lysate (RRL), hnRNP C binding to the 29-base element stabilized APP mRNA resulting in a 6-fold increase in APP protein production (2). The role of nucleolin was not determined in these experiments.

Nucleolin (C23) is a 110-kDa multifunctional phosphoprotein. It is an abundant nucleolar protein (3) found in the fibrillar centers and on organizer regions of metaphase chromosomes (4). Nucleolin plays a role in chromatin decondensation (5), the transcription and processing of rRNA (6–9), transcriptional regulation (10, 11), cell proliferation (12), differentiation and maintenance of neural tissue (13), apoptosis (14), nuclear/cytoplasmic shuttling (15), mRNA stability (16), and mRNP assembly and masking (17). Cell surface nucleolin has been reported to bind lipoproteins (18), laminin (13), growth factors (19), and the complement inhibitor, factor J (20). Central to nucleolin functions are RNA/DNA binding and helicase activities (21).

The cDNA for nucleolin has been cloned and codes for a 707-amino acid protein with at least three functional domains (3, 22). The 5'-flanking region and the first intron contain a high GC content similar to the housekeeping genes. The 5' promoter has one atypical TATA box (GTTA), one CCAAT box, three reverse complements of CCAAT (ATTGG), two pyrimidine-rich stretches, and numerous potential transcription factor-binding sites, whereas the 3'-UTR has five homology blocks in a 100-base region (23).

There are several distinct features in the amino acid sequence of nucleolin that enable this extraordinary protein to display such a vast array of functions (3, 7). At the amino-terminal end of the molecule, there are six (G/A/V)TP(G/A/V)KK(G/A/V)(G/A/V) repeats followed by several glutamic/aspartic acid stretches separated by basic sequences and four potential serine phosphorylation sites. The central region, a putative globular domain, contains alternating hydrophobic and hydrophilic stretches. There are four 90-residue repeats, each containing an RNP-like consensus sequence (24). The carboxyl terminus is glycine/arginine-rich with regularly spaced phenylalanine and dimethylarginine residues (25). The central 40-kDa domain of nucleolin containing the four RNA recognition motifs is responsible for the specificity of RNA binding, and the carboxyl-terminal domain enhances interaction but does not contribute to ligand specificity (26). The carboxyl-terminal domain contains an ATP-dependent duplex-

N-(6-phenylhexyl)-5-chloro-1-naphthalenesulfonamide; U0126, bis[2-aminophenylthio]butadiene; UTR, untranslated region.

Alzheimer's disease is characterized by the presence of senile plaques and neurofibrillary tangles in brain tissue. The major proteinaceous material in the senile plaques is β -amyloid, a 40–42-amino acid peptide derived from the amyloid precursor protein (APP).¹ Investigation of the molecular regulation of

* This work was supported by National Institutes of Health Grant RO1AG10675 (to J. S. M.) and NIA Research Service Award AG00213 from the National Institutes of Health (to C. J. W.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: University of Wisconsin Hospital and Clinics, Rm. K4/812, 600 Highland Ave., Madison, WI 53792. Tel.: 608-263-6043; Fax: 608-265-6215; E-mail: jsmalter@facstaff.wisc.edu.

¹ The abbreviations used are: APP, amyloid precursor protein; CK2, casein kinase 2; D609, tricyclodecan-9-yl xanthogenate; DRB, 5,6-dichlorobenzimidazole riboside; ECL, enhanced chemiluminescence; ERK, extracellular-regulated kinase; ET-18-OCH₃, 1-O-octadecyl-2-O-methyl-rac-glycero-3-phosphorylcholine; hnRNP, heterogeneous nuclear ribonucleoprotein; MAPK, mitogen-activated protein kinase; OAG, 1-oleoyl-2-acetyl-sn-glycerol (C18:1,[cis]-9/C2:0); PBMC, peripheral blood mononuclear cells; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; RRL, rabbit reticulocyte lysate; SC-9,

unwinding activity, and phosphorylation enhances this helix activity (21, 27).

We were initially interested in determining how nucleolin regulation might affect APP mRNA levels and stability. Preliminary studies suggested cytokine-mediated signaling through protein kinases altered nucleolin mRNA levels in PBMC. We found that activation of the extracellular-regulated kinase (ERK)-specific mitogen-activated protein kinase (MAPK) pathway significantly up-regulated nucleolin mRNA levels independently of protein kinase C (PKC). Phorbol 12-myristate 13-acetate (PMA) treatment also resulted in higher levels of full-length nucleolin protein and the disappearance of the 47-kDa nucleolin cleavage fragment. In gel mobility shift assays, lysates from phorbol ester and ionomycin-treated peripheral blood mononuclear cells (PBMC) contained nucleolin that bound to the 29-base instability element of APP mRNA. In RRL supplemented with nucleolin protein, APP mRNA decayed with a shortened half-life of 105 min but was indefinitely stable in RRL supplemented with globin. The loss of APP mRNA stability resulted in decreased APP production. Therefore, ERK activation stabilizes nucleolin mRNA resulting in increased nucleolin levels and accelerated decay of APP mRNA.

EXPERIMENTAL PROCEDURES

Materials—Protease inhibitor mixture (catalog number P2714), *Escherichia coli* tRNA, RNase TI, 4 α -phorbol, PMA, 1-oleoyl-2-acetyl-sn-glycerol (C18:1,*cis*-9/C2:0) (OAG), calcium ionophore A23187, ionomycin calcium salt, thapsigargin, dantrolene, forskolin, 1,9-dideoxyforskolin, cAMP, (R_p)-cAMPS, imipramine, wortmannin, 17 β -estradiol, corticosterone, and 5,6-dichlorobenzimidazole riboside (DRB) were from Sigma. *N*-(6-Phenylhexyl)-5-chloro-1-naphthalenesulfonamide (SC-9), tricyclodecan-9-yl xanthogenate (D609), and 1-*O*-octadecyl-2-*O*-methyl-rac-glycero-3-phosphorylcholine (Et-18-OCH₃) were from Calbiochem. Klenow enzyme, RNasin, bis[2-aminophenylthio]butadiene (U0126), and RRL were from Promega (Madison, WI). Lymphoprep density gradient medium and RPMI 1640 were from Life Technologies, Inc., and TRI-reagent was purchased from Molecular Research Center, Inc. (Cincinnati, OH). The enhanced chemiluminescence (ECL) Western blotting detection kit, [α -³²P]dCTP and L-[³⁵S]methionine were from Amersham Pharmacia Biotech; the nylon transfer membrane was from Fisher, and the QuikHyb hybridization solution and NucTrap probe purification columns were supplied by Stratagene (La Jolla, CA). The T7 and SP6 mMessage mMachine *in vitro* transcription kits and oligo(dT)-cellulose were purchased from Ambion, Inc. (Austin, TX).

PBMC Isolation and Stimulation, RNA Purification, and Northern Blotting—Peripheral blood was collected by phlebotomy from consenting, healthy laboratory personnel and was anticoagulated with heparin. The experimental protocol was approved by the University of Wisconsin Hospital Human Subjects Review Committee, which meets National Institutes of Health guidelines. PBMC were isolated by Ficoll-Paque density centrifugation, stimulated with various kinase effectors, and lysed in Tri-reagent as described previously.² RNA was electrophoresed on formaldehyde-agarose gels, transferred to nylon membranes, and hybridized with radiolabeled cDNA probes. The samples were assayed in at least triplicate, normalized to a control RNA (18 S or ribosomal protein S26 mRNA), and plotted as a percentage of total nucleolin mRNA. Error bars depict the standard error of the mean, and *p* values were calculated by the Student's *t* test.

Preparation of PBMC Cytoplasmic Lysates—Cytoplasmic lysates were prepared as described previously (29). Briefly, cultured PBMC (2 ml at 5×10^6 cells/ml) were scraped from tissue culture wells, spun at $2,000 \times g$ for 30 s in a Stratagene microfuge microcentrifuge, washed three times with ice-cold phosphate-buffered saline (PBS), and resuspended in 50 μ l of ice-cold buffer containing 25 mM Tris, pH 8, 0.1 mM EDTA, and 1 \times protease inhibitor mixture. The resuspended cells were lysed by five freeze (-80°C)/thaw (37°C) cycles and spun at $15,000 \times g$ for 15 min at 4°C . The supernatants (cytoplasmic lysates) were transferred to fresh tubes and frozen at -80°C . The protein concentration of the lysates was quantitatively determined with Bio-Rad protein assay dye reagent per the manufacturer's instructions.

Western Blot Analysis—Lysate (10 μ g) in a 12- μ l volume was mixed with 4 μ l (4 \times) SDS reducing buffer (62.5 mM Tris-HCl, pH 6.8, 10%

glycerol, 2% SDS, 5% β -mercaptoethanol, 0.06% bromphenol blue), boiled for 5 min, and analyzed on a 12% SDS-polyacrylamide gel. The gel was equilibrated in Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 20% methanol, 1.3 mM SDS, pH 9.2), and the proteins were transferred to 0.2- μ m pure nitrocellulose membrane by semi-dry electrophoretic transfer on a Bio-Rad Trans-Blot SD apparatus at 15 V for 45 min. The nitrocellulose membrane was blocked for 1 h in 5% nonfat dry milk and stained by ECL per the manufacturer's directions. The primary antibody was anti-nucleolin (1:1000) (kindly provided by Dr. Raymond Petryshyn, Children's National Medical Center, Washington, D. C.) and the secondary antibody was anti-rabbit horseradish peroxidase (1:2000). The membrane was exposed to x-ray film for 1 min.

Preparation of DNA Template and *In Vitro* Transcription—APP106 template was prepared by amplification of nucleotides 2415–2520 of the plasmid pT7APP751wt Δ HindIIIIT90 (2) with the primers 5'-CACAAATACGACTCAC-TATAGGGAACCTGAATTAATCCACA-3' (APP cDNA_(2415–2432)) and 5'-ACAGCTAAATTTCTTTACAGT-3' (APP cDNA_(2520–2501)) by PCR (1 min at 94°C , 1 min at 50°C , and 10 s at 72°C for 35 cycles). The 5' primer included a T7 RNA polymerase promoter sequence, which is underlined. The PCR product was extracted with Tris-saturated phenol/chloroform and precipitated with 0.1 volume of sodium acetate and 2 volumes of ethanol before gel purification on an 8% nondenaturing polyacrylamide gel. Radiolabeled RNA probes were prepared according to Promega's standard transcription protocol for T7 RNA polymerase with each labeling reaction containing 200 ng of APP106 template and 50 μ Ci of [α -³²P]UTP. Reactions were incubated for 60 min at 37°C and stopped by the addition of 2 units of RNase-free DNase I for 15 min at 37°C . Samples were extracted with water-saturated phenol/chloroform, and unincorporated isotope was removed by passage through NucTrap probe purification columns.

Gel Mobility Shift Assays—The binding reactions were performed similarly to the procedures used in Ref. 28. Briefly, 2 μ g of cytoplasmic lysates were incubated with 1×10^5 cpm of APP106 RNA probe in 10% glycerol, 15 mM HEPES, pH 8, 10 mM KCl, 1.0 mM dithiothreitol, 200 ng/ μ l *E. coli* tRNA, and 1 unit/ μ l RNasin in a total volume of 10 μ l for 10 min at 30°C . RNase TI (20 units in 1- μ l volume) was added, and samples were digested for 30 min at 37°C . Reactions were cross-linked on automatic for 5 min in a UV Stratallinker 2400 from Stratagene (La Jolla, CA) on ice prior to the addition of 3.5 μ l of 4 \times SDS reducing buffer, heat denaturation for 3 min at 100°C , and analysis on 12% SDS-polyacrylamide gels. The gels were dried and exposed to x-ray film or a PhosphorImager screen.

Plasmid Construction—The construction of pT7APP751wt Δ HindIIIIT90 has been described (2). The plasmid pSP κ β c containing the β -globin gene cloned between an SP6 promoter and a T stretch was provided by Richard Spritz (University of Wisconsin, Madison, WI), and the nucleolin gene in the pMAM plasmid was a gift from Meera Srivastava (Georgetown University School of Medicine, Washington, D. C.). The pMAM^{nucleolin} plasmid was digested with *Xho*I and *Nhe*I to liberate the nucleolin gene. The insert ends were blunted with Klenow and ligated with *Sma*I-digested pT7T90 transcription vector (provided by Jeff Ross, University of Wisconsin, Madison, WI) to generate pT7nucl^{coding}T90. The 3'-UTR of nucleolin cDNA was amplified from Jurkat genomic DNA with the primers 5'-ACGAAGTTTGAAT-AGCTTCT-3' (nucleolin cDNA_(2221–2240)) and 5'-GTAGGAAAAATGGTTT-TGT-3' (nucleolin cDNA_(2518–2499)) by PCR (1 min at 94°C , 1 min at 50°C , and 20 s at 72°C for 35 cycles). The ends were blunted with Klenow and ligated with *Sma*I-digested pUC-18 generating pUCnucl^{3'-UTR}. pUCnucl^{3'-UTR} was digested with *Eco*RI and *Xba*I to liberate the nucleolin 3'-UTR sequence, and the ends were blunted with T4 DNA polymerase and ligated with pT7nucl^{coding}T90 previously digested with *Eco*RI and blunted with T4 DNA polymerase. The resulting plasmid, pT7nucleolinT90, contained the entire nucleolin coding region, a short spacer, and the complete nucleolin 3'-UTR sequence cloned between a T7 promoter and a 90-base poly(T) stretch.

mRNA Synthesis and Purification—The plasmids pT7APP751 wt Δ HindIIIIT90 and pSP κ β c were linearized with *Hind*III, and the plasmid pT7nucleolinT90 was digested with *Eco*RV. The digests were extracted with phenol and chloroform, precipitated with 0.1 volume ammonium acetate and 2 volumes ethanol, resuspended in water to 0.5 μ g/ μ l, and used as templates (1 μ g each) for *in vitro* transcription with Ambion's T7 and Sp6 mMessage mMachine kits per the manufacturer's directions for 2 h at 37°C . The capped, polyadenylated mRNA reactions were digested with RNase-free DNase I for 15 min at 37°C , extracted with water-saturated phenol and chloroform, and purified by oligo(dT)-cellulose chromatography. The oligo(dT)-cellulose was equilibrated in high salt binding buffer containing 0.5 M NaCl, 20 mM Tris-Cl, pH 7.5, 1 mM EDTA. The mRNA was heat-denatured for 5 min at 65°C , chilled in ice, adjusted to high salt binding buffer conditions, and bound to the

² C. J. Westmark and J. S. Malter, submitted for publication.

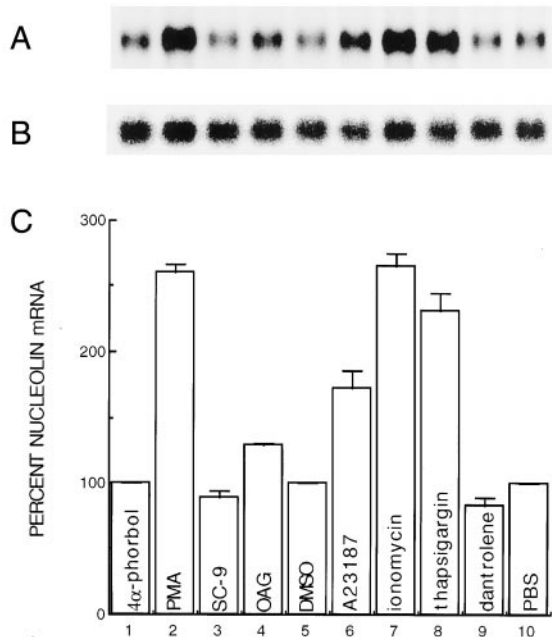


FIG. 1. MAPK and calcium signaling, but not PKC, participate in the regulation of nucleolin mRNA. PBMC were treated with 20 ng/ml 4 α -phorbol (lane 1), 20 ng/ml PMA (lane 2), 10 μ M SC-9 (lane 3), 200 μ M OAG (lane 4), 0.1% Me₂SO (DMSO, lane 5), 5 μ M A23187 (lane 6), 5 μ M ionomycin (lane 7), 50 nM thapsigargin (lane 8), 10 μ M dantrolene (lane 9), and 0.04% PBS (lane 10) for 3.25 h followed by a 15-min chase with DRB. Total RNA (6 μ g) was analyzed by Northern blotting. *A*, representative Northern blot of the nucleolin-specific hybridization signals. *B*, representative Northern blot of the S26-specific hybridization signals. *C*, histogram depicting nucleolin mRNA levels normalized to S26 and plotted as a percentage of total nucleolin mRNA. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: PMA, *p* = 0.0012; SC-9, *p* = 0.17; OAG, *p* = 0.00040; A23187, *p* = 0.028; ionomycin, *p* = 0.0028; thapsigargin, *p* = 0.0092; and dantrolene, *p* = 0.089.

oligo(dT)-cellulose for 30 min at ambient temperature with mixing. The resin was washed with high salt binding buffer followed by low salt binding buffer (0.1 M NaCl) and finally eluted with TE (pH 7.5) (65 °C). The purity of the mRNA was checked by agarose gel electrophoresis, and the concentration was measured by A₂₆₀.

Decay and Translation of APP751 mRNA in Nucleolin-supplemented RRL—RRL was programmed with 100 ng of heat-denatured globin *versus* nucleolin mRNA, and translation proceeded for 3.5 h at 30 °C per the manufacturer's directions. Aliquots of globin- and nucleolin-supplemented RRL were frozen at -80 °C. APP mRNA (50 ng) was incubated with 2 μ l of globin- *versus* nucleolin-supplemented RRL in 10- μ l reactions containing 10% glycerol, 15 mM HEPES, pH 8, 10 mM KCl, 1.0 mM dithiothreitol, and 200 ng/ μ l *E. coli* tRNA for 10 min at 30 °C. Translation was initiated by the addition of 33 μ l of fresh RRL, 0.5 μ l of amino acid mixture minus methionine, 0.5 μ l of amino acid mixture minus leucine, 1.4 μ l of KCl, and 4.6 μ l of water. Reactions (50 μ l each) were incubated at 30 °C, and 5- μ l aliquots were removed at 0, 30, 60, and 120 min. RNA was isolated with Tri-Reagent, and RNA pellets were dissolved in 25 μ l of formamide, and 5 μ l of each sample was analyzed on formaldehyde-agarose gels. For translation measurements, RRL reactions were similar to the RNA decay reactions although L-[³⁵S]methionine was included. Aliquots (2.5 μ l each) were removed at 0.5, 1, 2, and 3 h, diluted with 7.5 μ l of water, mixed with 3.5 μ l of 4 \times SDS reducing buffer, heated for 20 min at 60 °C, and analyzed on 12% SDS-polyacrylamide gels. The gels were dried and exposed to a PhosphorImager screen.

RESULTS

We examined the effect of several drugs that influence MAPK, PKC, protein kinase A (PKA), and phospholipase C (PLC) activity as well as calcium mobilization on the steady state level of nucleolin mRNA in PBMC. To investigate the role of PKC, cells were cultured for 3.25 h in the presence of the phorbol ester PMA. Compared with untreated controls, nucleo-

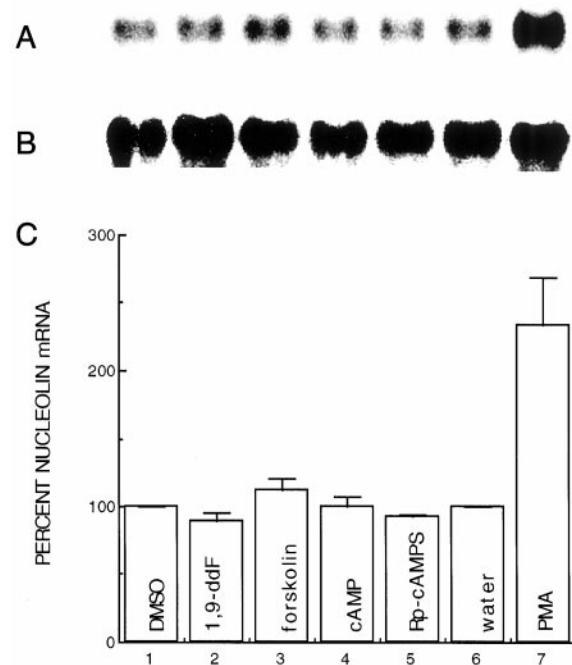


FIG. 2. PKA and adenylate cyclase agonists do not affect nucleolin mRNA levels in PBMC. Cells were treated 3.5 h with 0.1% Me₂SO (DMSO, lane 1), 13 μ M 1,9-dideoxyforskolin (1,9-ddF, lane 2), 13 μ M forskolin (lane 3), 142 μ M cAMP (lane 4), 29 μ M (R_p)-cAMPS (lane 5), 0.2% water (lane 6), and 20 ng/ml PMA (lane 7). After a 15-min chase with DRB, total RNA was isolated and analyzed by Northern blotting (6 μ g of total RNA per lane). *A*, representative Northern blot of the nucleolin-specific hybridization signals. *B*, representative Northern blot of the 18 S-specific hybridization signals. *C*, histogram depicting nucleolin mRNA levels normalized to 18 S and plotted as a percentage of total nucleolin mRNA. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: 1,9-dideoxyforskolin, *p* = 0.22; forskolin, *p* = 0.29; cAMP, *p* = 0.96; (R_p)-cAMPS, *p* = 0.065; PMA, *p* = 0.063.

lin mRNA levels increased by 2.5-fold (Fig. 1, lane 2). In all cases, nucleolin mRNA was a single 3.0-kilobase pair transcript. The inactive phorbol ester, 4 α -phorbol, did not stimulate nucleolin mRNA accumulation. Two additional activators of PKC, SC-9 and OAG, that stimulate calcium/phospholipid-dependent PKC were also tested (Fig. 1, lanes 3–4). SC-9 had no effect, whereas OAG, a diacylglycerol analog, had a small stimulatory effect (29%) on nucleolin mRNA levels. The PKC inhibitors bisindolylmaleimide I and staurosporine did not block the PMA-mediated increase in nucleolin mRNA levels indicating that PMA acts independently of PKC (data not shown).

The effect of several calcium mobilization drugs on nucleolin mRNA levels was next assessed. The ionophores A23187 and ionomycin, which transport calcium from the medium into cells, as well as thapsigargin, which causes the release of calcium from intracellular stores, all increased nucleolin mRNA levels 1.5–2.5-fold above unstimulated controls (Fig. 1, lane 6–8). Dantrolene (lane 9), which blocks intracellular calcium release, had no effect on nucleolin mRNA levels. Therefore, nucleolin mRNA levels were up-regulated in response to drugs that increase intracellular calcium concentrations via release from intracellular stores or import from the extracellular environment.

We examined the influence of several activators and inhibitors of PKA and PLC signal transduction on the steady state level of nucleolin mRNA in PBMC. Cyclic AMP, a known activator of PKA, and (R_p)-cAMPS, an inhibitor of PKA, did not change the steady state level of nucleolin mRNA (Fig. 2, lanes 4–5). Forskolin activates adenylate cyclase resulting in increased cAMP levels. 1,9-Dideoxyforskolin, a negative control

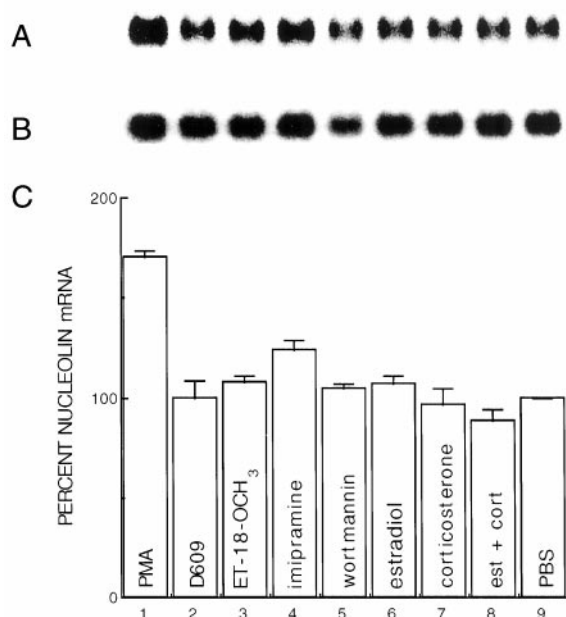


FIG. 3. PLC pathway agonists do not significantly alter nucleolin mRNA levels in PBMC. Cells were treated 4 h with 20 ng/ml PMA (lane 1), 19 μ M D609 (lane 2), 15 μ M ET-18-OCH₃ (lane 3), 100 μ M imipramine (lane 4), 10 nM wortmannin (lane 5), 100 nM 17 β -estradiol (lane 6), 1.5 μ M corticosterone (lane 7), 100 nM 17 β -estradiol with 1.5 μ M corticosterone (est + cort, lane 8), and 0.1% PBS (lane 9) followed by a 15-min chase with DRB. Total RNA (10 μ g per lane) was analyzed by Northern blotting. *A*, representative Northern blot of the nucleolin-specific hybridization signals. *B*, representative Northern blot of the S26-specific hybridization signals. *C*, histogram depicting nucleolin mRNA levels normalized to S26 and plotted as a percentage of total nucleolin mRNA. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: PMA, $p = 0.0029$; D609, $p = 1.0$; Et-18-OCH₃, $p = 0.11$; imipramine, $p = 0.036$; wortmannin, $p = 0.17$; 17 β -estradiol, $p = 0.23$; corticosterone, $p = 0.68$; and 17 β -estradiol + corticosterone, $p = 0.16$.

for forskolin, is unable to stimulate adenylate cyclase. Activation of the adenylate cyclase pathway with forskolin did not influence the steady state level of nucleolin mRNA in PBMC (Fig. 2, lane 3). We did observe a 24% increase in nucleolin mRNA levels upon treatment with imipramine, a drug that stimulates PLC in rat brain by a calcium-dependent process (29) (Fig. 3, lane 4). We did not observe any significant change in nucleolin mRNA levels with D-609, Et-18-OCH₃, or wortmannin (Fig. 3, lanes 2, 3 and 5), drugs that inhibit phosphatidylcholine-specific PLC, phosphatidylinositol-specific PLC, and phosphatidylinositol 3-kinase, respectively. The steroid hormones, estrogen and corticosterone, also did not affect nucleolin mRNA levels (Fig. 3, lanes 7 and 8). Therefore, the imipramine-mediated effects suggest that the PLC pathway partially modulates nucleolin mRNA levels in PBMC.

PMA is a known activator of the MAPK signal transduction pathway, so we next assessed the ability of U0126 to block the PMA-mediated increase in nucleolin mRNA levels (Fig. 4). Cells were pretreated with 10 μ M U0126 for 15 min prior to the addition of 20 ng/ml PMA for 3 h. The cultures were spiked with 5 μ M U0126 after 1 and 2 h with PMA to maintain anti-MEK activity. U0126 blocked the PMA-mediated increase in nucleolin mRNA levels (Fig. 4, lane 3) pointing to the ERK-specific MAPK pathway as a major player in regulating nucleolin mRNA levels.

Nucleolin mRNA accumulation could be secondary to increased transcription, decreased degradation, or both. Thus, nucleolin mRNA decay was measured in resting or PMA-treated PBMC. In resting PBMC, the half-life of nucleolin mRNA was 1.8 h (Fig. 5), which increased almost 2-fold ($t_{1/2}$ =

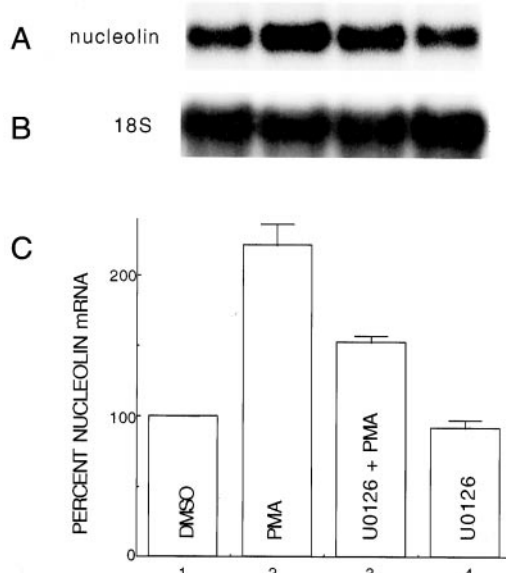


FIG. 4. U0126 partially blocks the PMA-mediated increase in nucleolin mRNA levels. PBMC were treated with 0.1% Me₂SO (DMSO, lane 1), 20 ng/ml PMA (lane 2), 20 μ M U0126 plus 20 ng/ml PMA (lane 3), or 20 μ M U0126 (lane 4). The U0126, or for controls an equal quantity of Me₂SO vehicle, was added to the cells in increments to maintain activity. The cells were pretreated for 15 min with 10 μ M U0126 followed by 5 μ M additions after 1 and 2 h with +/- PMA. DRB was added for 15 min, and total RNA (11 μ g per lane) was isolated and analyzed as described under "Experimental Procedures." *A*, representative Northern blot of the nucleolin-specific hybridization signals. *B*, representative Northern blot of the 18 S-specific hybridization signals. *C*, bar graph depicting nucleolin mRNA levels normalized to 18 S and plotted as a percentage of total nucleolin mRNA. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: PMA, $p = 0.016$; U0126 + PMA, $p = 0.0092$; U0126, $p = 0.28$.

3.2 h) after treatment for 3.5 h with PMA. Therefore, nucleolin mRNA accumulation after ERK activation can be accounted for by enhanced stability of the message.

As mentioned earlier, nucleolin protein has been implicated in many functions including APP mRNA stability. Thus, we examined cell lysates for nucleolin expression by Western blotting and nucleolin binding activity by RNA gel mobility shift assays. There was a time-dependent increase in nucleolin protein levels upon PMA treatment (Fig. 6). On ECL-stained Western blots probed with a polyclonal anti-nucleolin antibody, we observed two prominent nucleolin bands at 47 and 65 kDa in unstimulated PBMC. After PMA stimulation for 15 min, there was an increase in nucleolin fragments of ~70 kDa, and after 1 h, an increase in the 80-kDa nucleolin fragment. We observed full-length nucleolin protein (100 kDa) after 2 h of PMA treatment which continued to increase for several hours. As full-length nucleolin increased there was a decrease in the 47-kDa nucleolin cleavage product. RNA mobility shifts with radiolabeled APP RNA demonstrated a 2.8-fold increase in nucleolin binding after only 20 min of PMA treatment and a 5.1-fold increase by 135 min (Fig. 7). Our laboratory has previously demonstrated that the 70-kDa nucleolin polypeptide is responsible for the 84-kDa nucleolin/29-base element RNA-protein complex (1). In unstimulated cells, there were faint nucleolin-APP RNA complexes in the 60–70-kDa range (Fig. 7, lane 1) which were previously observed only in stimulated cells (16). These lower molecular weight complexes are not due to cell stimulation during the PBMC isolation procedure but rather are a consequence of including RNasin in the binding buffer. We have found an RNase A-like activity in PBMC that is inactivated by PMA or ionophore stimulation of the cells (data not shown). These complexes were specific for APP mRNA since

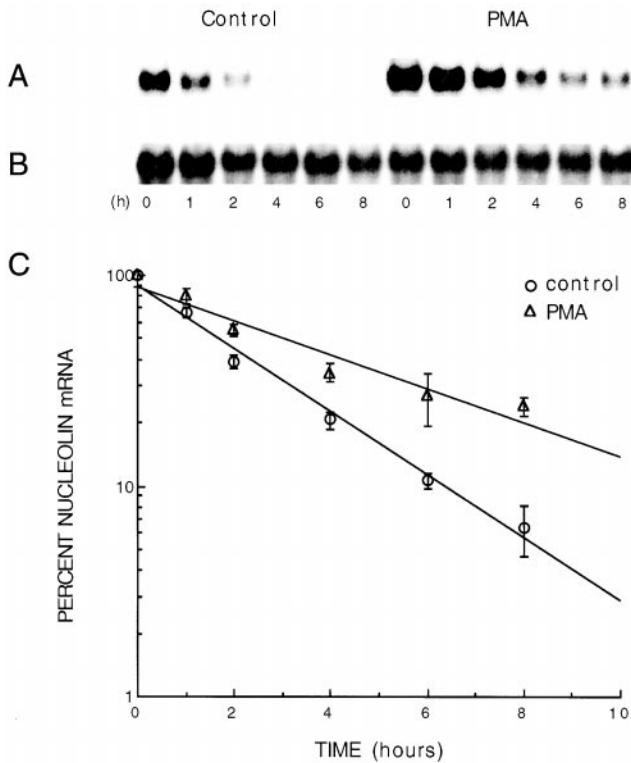


FIG. 5. PMA up-regulates and stabilizes nucleolin mRNA. Cells were cultured in the presence of 0.002% ethanol (lanes 1–6) or 20 ng/ml PMA (lanes 7–12) for 3.5 h. Transcription was stopped by the addition of 20 μ g/ml DRB, and after 15 min, total RNA was isolated at the indicated time points (0, 1, 2, 4, 6, and 8 h), and 5 μ g of total RNA per lane was analyzed by Northern blotting. A, representative Northern blot of the nucleolin-specific hybridization signals. B, representative Northern blot of the 18 S-specific hybridization signals. C, line graph depicting the percentage of nucleolin mRNA versus time. The circles represent nucleolin mRNA levels in the presence of ethanol alone (vehicle), and the triangles represent the nucleolin mRNA levels in the presence of PMA. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: at 0 h, $p = 0.015$; at 1 h, $p = 0.00026$; at 2 h, $p = 0.00013$; at 4 h, $p = 0.00029$; at 6 h, $p = 0.030$; at 8 h, $p = 0.00015$.

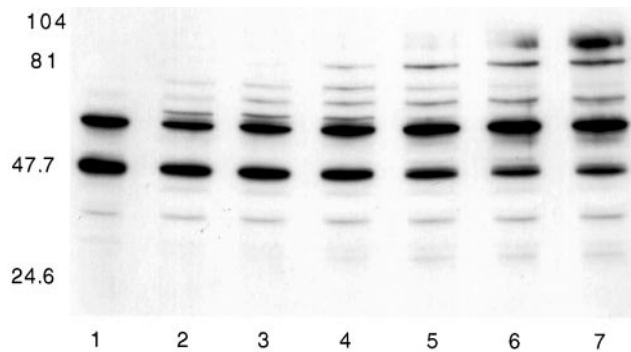


FIG. 6. PMA stimulation increases the production of nucleolin protein in PBMC. Cells were treated for 15 min with 0.002% ethanol (lane 1) or 20 ng/ml PMA for 15 min (lane 2), 30 min (lane 3), 1 (lane 4), 2 (lane 5), 3 (lane 6), and 4 h (lane 7). Cell lysates (10 μ g per lane) were analyzed by SDS-polyacrylamide gel electrophoresis on a 12% gel, transferred to nitrocellulose membrane, and stained by ECL with anti-nucleolin serum.

they could not be detected in gel mobility shift assays with an APP RNA probe containing a randomized 29-base element (data not shown).

Cell activation with PMA, A23187, and ionomycin caused similar increases in the 84-kDa nucleolin-APP mRNA complexes (Fig. 8), whereas PKC, PKA, or PLC activation had no

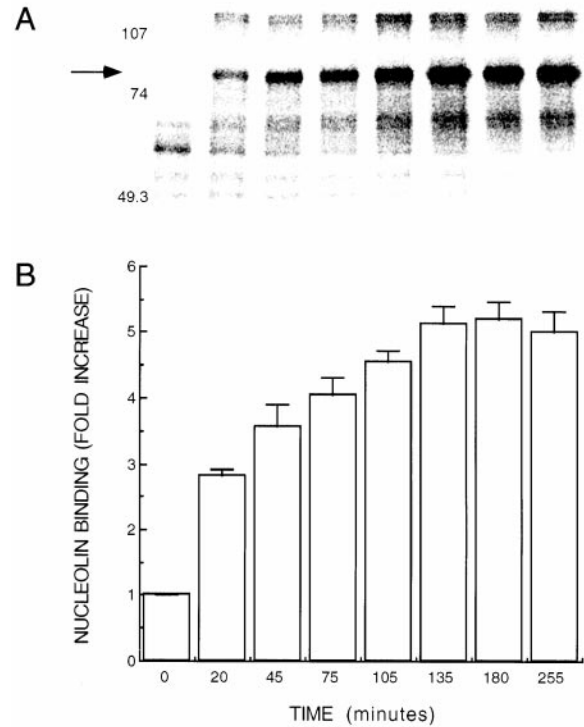


FIG. 7. PMA stimulates the ability of the 70-kDa nucleolin polypeptide to bind to the 3'-UTR of APP mRNA. Cells were treated for 20 min with 0.002% ethanol (lane 1) or 20 ng/ml PMA for 20 (lane 2), 45 (lane 3), 75 min (lane 4), 105 (lane 5), 135 (lane 6), 180 (lane 7), and 255 min (lane 8). A, representative gel mobility shift assay. The arrow denotes the 84-kDa shift complex. B, histogram depicting the fold increase in nucleolin binding versus stimulation time with PMA. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: at 20 min, $p = 0.0036$; at 45 min, $p = 0.016$; at 75 min, $p = 0.0066$; at 105 min, $p = 0.0025$; at 135 min, $p = 0.0040$; at 180 min, $p = 0.0047$; at 255 min, $p = 0.0065$.

effect. Treatment of cells with U0126 prior to the addition of PMA blocked shift formation (Fig. 8E, lane 3). Thus drugs that activated ERK and calcium second messenger pathways increased nucleolin mRNA, protein, and APP RNA binding activity.

Nucleolin is a well established RNA-binding protein with RNA helicase activity (21). We assessed the role nucleolin plays in APP mRNA stability and translation in an RRL translation system. Globin (control) or nucleolin mRNA was translated in RRL and subsequently incubated with APP mRNA as described under "Experimental Procedures." Fresh RRL was then transferred to the preprogrammed APP mRNA, and translation and mRNA decay were measured by Northern blot analysis. Preincubation of the APP mRNA template with nucleolin increased decay of the message ($t_{1/2} = 105$ min) (Fig. 9). Nucleolin also accelerated the decay of an APP mRNA containing a mutated 29-base element (APP^{mut} mRNA) and decay of globin mRNA (data not shown). These mRNAs were all stable in RRL when preprogrammed with globin. Therefore, although nucleolin does bind to the 29-base instability element, it enhanced decay of APP mRNA independently of the element.

Nucleolin is a component of translation inhibitory particles (17). Translation of APP mRNA in RRL was measured by radioactive methionine incorporation. APP mRNA preprogrammed with nucleolin was translated at 52% of the level as template preprogrammed with globin (Fig. 10, 1 h). Similarly, translation of APP^{mut} and globin mRNAs preprogrammed with nucleolin was also decreased (data not shown). Thus, APP production was compromised largely by accelerated decay of its coding mRNA.

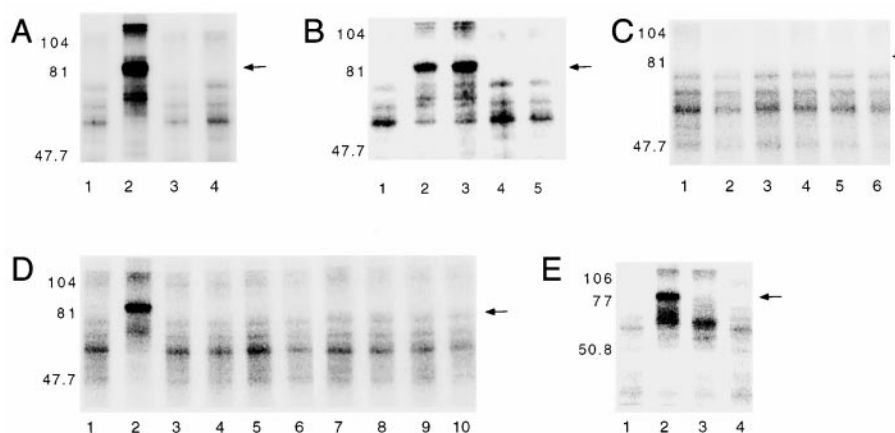


FIG. 8. PMA and ionophores stimulate the ability of nucleolin to bind to the 3'-UTR of APP mRNA. Cells were treated with the indicated drugs at the concentrations noted in Fig. legends 1–3. *A*, gel mobility shift assays after PKC activators. Cells were stimulated with 4 α -phorbol (lane 1), PMA (lane 2), SC-9 (lane 3), and OAG (lane 4). *B*, gel mobility shift assays after calcium mobilization drugs. Cells were stimulated with Me₂SO (lane 1), A23187 (lane 2), ionomycin (lane 3), dantrolene (lane 4), and PBS (lane 5). *C*, gel mobility shift assays after PKA/adenylate cyclase agonists. PBMC were treated with Me₂SO (lane 1), 1,9 dideoxyforskolin (lane 2), forskolin (lane 3), cAMP (lane 4), (R_p)-cAMPS (lane 5), and water (lane 6). *D*, gel mobility shift assays after PLC effectors. Cells were treated with ethanol (lane 1), PMA (lane 2), D609 (lane 3), ET-18-OCH₃ (lane 4), imipramine (lane 5), wortmannin (lane 6), 17 β -estradiol (lane 7), corticosterone (lane 8), 17 β -estradiol with corticosterone (lane 9), and PBS (lane 10). *E*, gel mobility shift assays after ERK agonists. Cells were pretreated with 10 μ M U0126 for 15 min prior to the addition of PMA for 3 h with Me₂SO (lane 1), PMA (lane 2), U0126 + PMA (lane 3) and U0126 (lane 4). The arrow denotes the 84-kDa shift complex.

DISCUSSION

Our data indicate that activation of the MAPK or calcium second messenger pathways in PBMC results in a substantial accumulation of nucleolin mRNA. Drugs that activate/inhibit the PKA signal transduction pathway did not alter the steady state level of nucleolin mRNA, whereas PKC activation with OAG or PLC activation with imipramine had only modest effects. PMA stimulation of the cells resulted in posttranscriptional stabilization of the nucleolin message.

It was previously demonstrated that nucleolin mRNA levels increased in response to PHA in lymphocytes (30), serum stimulation in HeLa cells (31), vitamin A in monkey tracheobronchial epithelial cells (32), interleukin-2 in the C30.1 T cell line (33), and during liver regeneration in rat hepatocytes (34), whereas nucleolin mRNA levels were down-regulated during differentiation of human neuroblastoma cells with dibutyryl cAMP and/or retinoic acid (35) and by glucocorticoids in murine lymphosarcoma cells (36). In this study, we report that phorbol ester activation of ERK in PBMC increased nucleolin mRNA levels, and this increase was predominantly due to posttranscriptional stabilization of nucleolin mRNA. The half-life of nucleolin mRNA in resting PBMC was 1.8 h and increased to 3.2 h in PMA-treated cells. The cis acting domains responsible for regulated nucleolin mRNA stability are unknown. However, a comparison of the human, mouse, and hamster genomes reveals the presence of five homology blocks (nucleotides 2250–2258, 2268–2289, 2296–2302, 2305–2318, and 2321–2329) in the 3'-UTR within a 100-base region immediately following the stop codon (23). The first homology block is composed entirely of pyrimidine residues and is found within a uridine/cytidine-rich area. RNA-binding proteins with known specificities for pyrimidine-rich regions, such as pyrimidine tract binding protein, hnRNP C, or nucleolin itself, may bind to this region and protect the mRNA from RNase attack.

ERK signaling in PBMC resulted in increased nucleolin protein levels and binding activity to the 3'-UTR of APP mRNA. Nucleolin and hnRNP C form multiple RNA-protein complexes with the 29-base instability element situated ~200 bases downstream from the stop codon of APP mRNA (1). The 70-kDa nucleolin polypeptide is a constituent of previously identified 84-, 104-, and 140-kDa APP RNA-nucleolin complexes. The 47-

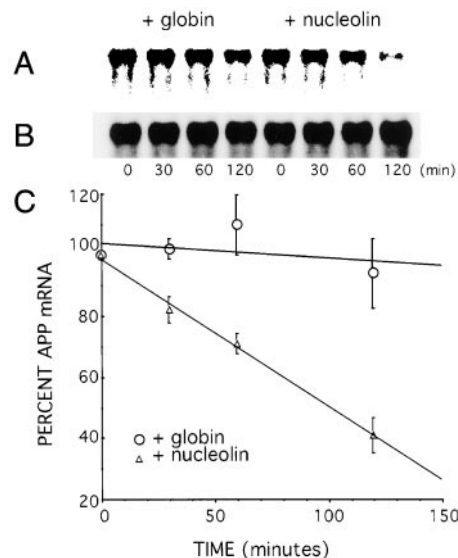


FIG. 9. Nucleolin destabilizes APP mRNA in an RRL translation system. *In vitro* transcribed, capped, polyadenylated nucleolin, and globin mRNAs (100 ng) were translated in RRL for 3.5 h. A 2- μ l aliquot was incubated with 50 ng of APP mRNA for 10 min before translation was initiated by the addition of RRL as described under "Experimental Procedures." Aliquots (5 μ l) were removed at 0, 30, 60, and 120 min followed by RNA isolation and Northern blotting. *A*, representative Northern blot of APP-specific hybridization signals. *B*, representative Northern blot of 18 S-specific hybridization signals. *C*, line graph depicting the percentage of APP mRNA remaining versus time. The circles represent APP mRNA levels after preincubation with globin, and the triangles represent APP message levels in the presence of nucleolin. The error bars represent the S.E. for triplicate samples. Student's *t* test results are as follows: at 30 min, $p = 0.021$; at 60 min, $p = 0.019$; at 120 min, $p = 0.014$.

and 48-kDa nucleolin peptides constitute the 65-, 73-, 90-, and 104-kDa complexes, suggesting protein homo- and heterodimers assemble on APP RNA (1). The 70-, 48-, and 47-kDa polypeptides all contain the carboxyl-terminal RNP domains. Since we observed increased binding of the 70-kDa nucleolin fragment to APP mRNA as more full-length nucleolin was produced by PMA-stimulated cells, and nucleolin exhibits increased susceptibility to proteolysis upon binding nucleic acid

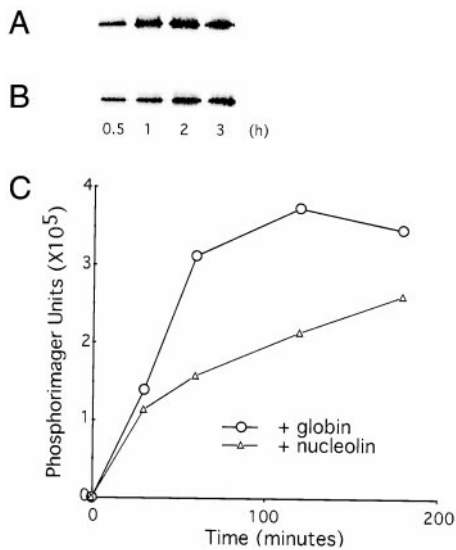


FIG. 10. Nucleolin decreases APP translation in a RRL translation system. Reactions were similar to Fig. 9 except for the addition of L-[³⁵S]methionine. Aliquots (2.5 μ l) were removed after 0.5 (lane 1), 1 (lane 2), 2 (lane 3), and 3 h (lane 4) followed by SDS-polyacrylamide gel electrophoresis. A, APP translation in presence of globin. B, APP translation in presence of nucleolin. C, line graph depicting APP translation level versus time. These data are representative of three experiments.

(37), we cannot exclude the possibility that full-length nucleolin binds to the 29-base instability element and then undergoes proteolysis of its amino-terminal domain. The 70-kDa nucleolin polypeptide as well as hnRNP C bind to the 5'-region of the 29-base element, whereas the smaller nucleolin fragments likely interact with the 3'-region (1, 16). The transition from the lower molecular weight nucleolin complexes we observe in unstimulated cells to the predominant 84-kDa complex in PMA-treated cells coincides with decreased stability of APP mRNA,² suggesting that mRNA stability is dependent on which nucleolin polypeptides bind to the 29-base element. These data also supports a model in which there is competition between nucleolin and hnRNP C for binding to the 5'-region of the cis element.

Phosphorylation and cleavage likely regulate the activity of nucleolin. Amino-terminal phosphorylation enhanced binding to histone H1 and chromatin condensation (5). Nucleolin is phosphorylated on serine residues by casein kinase 2 (CK2) (38, 39) during interphase, suggesting phosphorylation plays a role in the control of rDNA transcription, but nucleolin is phosphorylated on threonine residues by cdc2 kinase during mitosis, which has been linked to mitotic reorganization of nucleolar chromatin (40, 41). CK2 activity increases during cellular growth and declines when cells reach quiescence in parallel with the phosphorylation status of nucleolin (42) suggesting that CK2 may be an upstream effector of nucleolin. This idea is strengthened by the fact that the α -subunit of CK2 is physically associated with nucleolin (43). Nucleolin is present as several fragments of 100, 70, 60, and 50 kDa in nondividing cells (44), whereas the major form of the protein is full-length in proliferating cells (110 kDa). Nucleolin can be cleaved by proteases, for instance granzyme A (45), but possesses an intrinsic self-cleaving activity (46). Autocleavage is facilitated by phosphorylation and generates a highly phosphorylated 30- and a 72-kDa peptide (47). Upon cell proliferation, the self-cleaving activity is diminished due to nuclear proteolytic inhibitor(s) that stabilize the full-length nucleolin (44, 48). These data suggest that signaling cascades involving CK2 or cdc2 kinase control nucleolin cleavage and activity.

Characterization of the nucleic acid-binding sites of nucleolin

revealed a large array of DNA and RNA motifs including the pre-mRNA 3' splice site sequence r(UUAG/G), the human telomeric DNA sequence d(TTAGGG)_n (49), and the 3'-UTR of poliovirus RNA (50). The exact poliovirus 3'-UTR sequence has not been determined but may involve nucleotides 7365–7373 (CAUUUUAGU) which are very similar to the central portion of the APP 29-base element (16, 50). The nucleolin recognition element is an 18-base stem-loop containing the sequence UC-CCGA (51). Mutations in this sequence prevented the specific interaction between nucleolin and the RNA target (52). These data suggest that nucleolin preferentially binds short stretches of C/U nucleotides.

Our laboratory has demonstrated that the proteins hnRNP C and nucleolin bind to the 29-base instability element in the 3'-UTR of APP mRNA (1, 16). We have also shown that hnRNP C binding to this element stabilizes the APP message and increases protein production in RRL (2). In PBMC, APP mRNA underwent biphasic decay upon ERK activation with a rapid, initial fall in message quantity followed by prolonged stability.² We have proposed that the stable phase was a consequence of hnRNP C binding to the 29-base instability element that prevented RNase attack. In this paper, we demonstrate that nucleolin mRNA was up-regulated in PBMC via PMA or calcium ionophore treatment. The phorbol ester-mediated stabilization of nucleolin mRNA was accompanied by accumulation and decreased processing of nucleolin protein. The initial drop in the biphasic decay of APP mRNA corresponds temporally with increased nucleolin production. In RRL, APP mRNA preprogrammed with nucleolin decays with a half-life of 105 min but is stable in globin-supplemented RRL. Declines in APP message levels correspond with decreased translation.

The Gly-rich carboxyl-terminal domain of nucleolin contains DNA and RNA helicase activity that is modulated by phosphorylation (21). Another DNA and RNA helicase, G3BP, has sequence similarity with the carboxyl-terminal portion of nucleolin and is an element of the Ras signal transduction pathway (53). Collectively our data support a model in which nucleolin is up-regulated in response to ERK activation and then unwinds APP mRNA allowing RNase attack. Although nucleolin binds to the pyrimidine-rich 29-base element, the destabilizing effect of this protein on APP mRNA appears independent of the cis element since APP^{mut} mRNA also decays rapidly.

These findings are meaningful in that APP undergoes processing by β - and γ -secretases to produce β -amyloid, a 40–42-amino acid peptide found in the senile plaques characteristic of Alzheimer's disease and Down's syndrome. Significant levels of nucleolin have been found in mature brain and in differentiating neuronal cells (13). Thus, dysregulation of this multifunctional protein could play an important role in regulating APP mRNA stability, APP levels, and β -amyloid production.

Acknowledgments—We thank Dr. Raymond Petryshyn (Children's National Medical Center, Washington D. C.) for anti-nucleolin polyclonal antibody; Dr. Jeff Ross and Dr. Richard Spritz (University of Wisconsin, Madison, WI) for the plasmids pT7T90 and pSP κ β c, respectively; Dr. Meera Srivastava (Georgetown University School of Medicine, Washington D. C.) for the plasmid pMAM^{nucleolin}; the nursing staff (Infusion Center, University of Wisconsin Hospital) for drawing blood from volunteer donors; Dr. William Rehrauer (Molecular Diagnostics Laboratory, University of Wisconsin Hospital) for automated sequencing of plasmids; and members of the laboratory for their thoughtful comments.

REFERENCES

- Zaidi, S. H., and Malter, J. S. (1995) *J. Biol. Chem.* **270**, 17292–17298
- Rajagopalan, L. E., Westmark, C. J., Jarzembowski, J. A., and Malter, J. S. (1998) *Nucleic Acids Res.* **26**, 3418–3423
- Lapeyre, B., Bourbon, H., and Amalric, F. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1472–1476
- Lischwe, M. A., Richards, R. L., Busch, R. K., and Busch, H. (1981) *Exp. Cell*

- Res.* **136**, 101–109
5. Erard, M. S., Belenguer, P., Caizergues-Ferrer, M., Pantaloni, A., and Amalric, F. (1988) *Eur. J. Biochem.* **175**, 525–530
 6. Eghyazi, E., Pigon, A., Chang, J. H., Ghaffari, S. H., Dreesen, T. D., Wellman, S. E., Case, S. T., and Olson, M. O. (1988) *Exp. Cell Res.* **178**, 264–272
 7. Jordan, G. (1987) *Nature* **329**, 489–490
 8. Ginisty, H., Amalric, F., and Bouvet, P. (1998) *EMBO J.* **17**, 1476–1486
 9. Bouvet, P., Diaz, J. J., Kindbeiter, K., Madjar, J. J., and Amalric, F. (1998) *J. Biol. Chem.* **273**, 19025–19029
 10. Belenguer, P., Baldin, V., Mathieu, C., Prats, H., Bensaid, M., Bouche, G., and Amalric, F. (1989) *Nucleic Acids Res.* **17**, 6625–6636
 11. Yang, T. H., Tsai, W. H., Lee, Y. M., Lei, H. Y., Lai, M. Y., Chen, D. S., Yeh, N. H., and Lee, S. C. (1994) *Mol. Cell. Biol.* **14**, 6068–6074
 12. Derenzini, M., Sirri, V., Trere, D., and Ochs, R. L. (1995) *Lab. Invest.* **73**, 497–502
 13. Kibbey, M. C., Johnson, B., Petryshyn, R., Jucker, M., and Kleinman, H. K. (1995) *J. Neurosci. Res.* **42**, 314–322
 14. Brockstedt, E., Rickers, A., Kostka, S., Laubersheimer, A., Dorken, B., Wittmann-Liebold, B., Bommert, K., and Otto, A. (1998) *J. Biol. Chem.* **273**, 28057–28064
 15. Borer, R. A., Lehner, C. F., Eppenberger, H. M., and Nigg, E. A. (1989) *Cell* **56**, 379–390
 16. Zaidi, S. H., Denman, R., and Malter, J. S. (1994) *J. Biol. Chem.* **269**, 24000–24006
 17. Yurkova, M. S., and Murray, M. T. (1997) *J. Biol. Chem.* **272**, 10870–10876
 18. Semenkovich, C. F., Ostlund, R. E., Jr., Olson, M. O., and Yang, J. W. (1990) *Biochemistry* **29**, 9708–9713
 19. Take, M., Tsutsui, J., Obama, H., Ozawa, M., Nakayama, T., Maruyama, I., Arima, T., and Muramatsu, T. (1994) *J. Biochem. (Tokyo)* **116**, 1063–1068
 20. Larrucea, S., Gonzalez-Rubio, C., Cambronero, R., Ballou, B., Bonay, P., Lopez-Granados, E., Bouvet, P., Fontan, G., Fresno, M., and Lopez-Trascasa, M. (1998) *J. Biol. Chem.* **273**, 31718–31725
 21. Tuteja, N., Huang, N. W., Skopac, D., Tuteja, R., Hrvatic, S., Zhang, J., Pongor, S., Joseph, G., Faucher, C., Amalric, F., and Falaschi, A. (1995) *Gene (Amst.)* **160**, 143–148
 22. Srivastava, M., Fleming, P. J., Pollard, H. B., and Burns, A. L. (1989) *FEBS Lett.* **250**, 99–105
 23. Srivastava, M., McBride, O. W., Fleming, P. J., Pollard, H. B., and Burns, A. L. (1990) *J. Biol. Chem.* **265**, 14922–14931
 24. Bugler, B., Bourbon, H., Lapeyre, B., Wallace, M. O., Chang, J. H., Amalric, F., and Olson, M. O. (1987) *J. Biol. Chem.* **262**, 10922–10925
 25. Lapeyre, B., Amalric, F., Ghaffari, S. H., Rao, S. V., Dumbar, T. S., and Olson, M. O. (1986) *J. Biol. Chem.* **261**, 9167–9173
 26. Ghisolfi, L., Kharrat, A., Joseph, G., Amalric, F., and Erard, M. (1992) *Eur. J. Biochem.* **209**, 541–548
 27. Ghisolfi, L., Joseph, G., Amalric, F., and Erard, M. (1992) *J. Biol. Chem.* **267**, 2955–2959
 28. Malter, J. S. (1989) *Science* **246**, 664–666
 29. Fukuda, H., Nishida, A., Saito, H., Shimizu, M., and Yamawaki, S. (1994) *Neurochem. Int.* **25**, 567–571
 30. Mehes, G., and Pajor, L. (1995) *Cell Proliferation* **28**, 329–336
 31. Konishi, T., Karasaki, Y., Nomoto, M., Ohmori, H., Shibata, K., Abe, T., Shimizu, K., Itoh, H., and Higashi, K. (1995) *J. Biochem. (Tokyo)* **117**, 1170–1177
 32. Reddy, P. M., An, G., Di, Y. P., Zhao, Y. H., and Wu, R. (1996) *Am. J. Respir. Cell Mol. Biol.* **15**, 398–403
 33. Herblot, S., Chastagner, P., Samady, L., Moreau, J. L., Demaison, C., Frousard, P., Liu, X., Bonnet, J., and Theze, J. (1999) *J. Immunol.* **162**, 3280–3288
 34. Ohmori, H., Murakami, T., Furutani, A., Higashi, K., Hirano, H., Gotoh, S., Kuroiwa, A., Masui, A., Nakamura, T., and Amalric, F. (1990) *Exp. Cell Res.* **189**, 227–232
 35. Murakami, T., Ohmori, H., Gotoh, S., Tsuda, T., Ohya, R., Akiya, S., and Higashi, K. (1991) *J. Biochem. (Tokyo)* **110**, 146–150
 36. Meyuhos, O., Baldin, V., Bouche, G., and Amalric, F. (1990) *Biochim. Biophys. Acta* **1049**, 38–44
 37. Olson, M. O., Kirstein, M. N., and Wallace, M. O. (1990) *Biochemistry* **29**, 5682–5686
 38. Caizergues-Ferrer, M., Belenguer, P., Lapeyre, B., Amalric, F., Wallace, M. O., and Olson, M. O. (1987) *Biochemistry* **26**, 7876–7883
 39. Csermely, P., Schnaider, T., Cheatham, B., Olson, M. O., and Kahn, C. R. (1993) *J. Biol. Chem.* **268**, 9747–9752
 40. Belenguer, P., Caizergues-Ferrer, M., Labbe, J. C., Doree, M., and Amalric, F. (1990) *Mol. Cell. Biol.* **10**, 3607–3618
 41. Peter, M., Nakagawa, J., Doree, M., Labbe, J. C., and Nigg, E. A. (1990) *Cell* **60**, 791–801
 42. Schneider, H. R., and Issinger, O. G. (1989) *Biochim. Biophys. Acta* **1014**, 98–100
 43. Li, D., Dobrowolska, G., and Krebs, E. G. (1996) *J. Biol. Chem.* **271**, 15662–15668
 44. Fang, S. H., and Yeh, N. H. (1993) *Exp. Cell Res.* **208**, 48–53
 45. Pasternack, M. S., Bleier, K. J., and McInerney, T. N. (1991) *J. Biol. Chem.* **266**, 14703–14708
 46. Bourbon, H. M., Bugler, B., Caizergues-Ferrer, M., Amalric, F., and Zalta, J. P. (1983) *Mol. Biol. Rep.* **9**, 39–47
 47. Warrenner, P., and Petryshyn, R. (1991) *Biochem. Biophys. Res. Commun.* **180**, 716–723
 48. Chen, C. M., Chiang, S. Y., and Yeh, N. H. (1991) *J. Biol. Chem.* **266**, 7754–7758
 49. Ishikawa, F., Matunis, M. J., Dreyfuss, G., and Cech, T. R. (1993) *Mol. Cell. Biol.* **13**, 4301–4310
 50. Waggoner, S., and Sarnow, P. (1998) *J. Virol.* **72**, 6699–6709
 51. Serin, G., Joseph, G., Faucher, C., Ghisolfi, L., Bouche, G., Amalric, F., and Bouvet, P. (1996) *Biochimie (Paris)* **78**, 530–538
 52. Ghisolfi-Nieto, L., Joseph, G., Puvion-Dutilleul, F., Amalric, F., and Bouvet, P. (1996) *J. Mol. Biol.* **260**, 34–53
 53. Costa, M., Ochem, A., Staub, A., and Falaschi, A. (1999) *Nucleic Acids Res.* **27**, 817–821