Formation of a Complex between Nucleolin and Replication Protein A after Cell Stress Prevents Initiation of DNA Replication

Yaron Daniely and James A. Borowiec

Department of Biochemistry, and Kaplan Comprehensive Cancer Center, New York University School of Medicine, New York, New York 10016

Abstract. We used a biochemical screen to identify nucleolin, a key factor in ribosome biogenesis, as a high-affinity binding partner for the heterotrimeric human replication protein A (hRPA). Binding studies in vitro demonstrated that the two proteins physically interact, with nucleolin using an unusual contact with the small hRPA subunit. Nucleolin significantly inhibited both simian virus 40 (SV-40) origin unwinding and SV-40 DNA replication in vitro, likely by nucleolin preventing hRPA from productive interaction with the SV-40 initiation complex. In vivo, use of epifluorescence and confocal microscopy showed that heat shock caused a dramatic redistribution of nucleolin from the nucleolus to the nucleoplasm. Nucleolin relocalization was concomitant with a tenfold increase in nucleolin–hRPA complex formation. The relocalized nucleolin significantly overlapped with the position of hRPA, but only poorly with sites of ongoing DNA synthesis. We suggest that the induced nucleolin–hRPA interaction signifies a novel mechanism that represses chromosomal replication after cell stress.

Key words: replication protein A • nucleolin • nucleolus • SV-40 • heat shock

Introduction

Replication protein A (RPA)[1] is a pivotal factor in eukaryotic DNA metabolism, wherein it acts as the primary single-stranded DNA (ssDNA)-binding protein (SSB) (Iftode et al., 1999). In addition to having an important function in the stabilization of ssDNA, RPA also facilitates various DNA transactions through physical interactions with other metabolic factors. Characterization of novel RPA-interacting factors has revealed additional pathways in which RPA participates and over which RPA can exert modulatory effects. A widespread network of functionally significant RPA interactions has recently become apparent.

In common with RPA from other organisms, human RPA (hRPA) is a heterotrimer composed of 70- (hRPA1), 29- (hRPA2), and 14-kD (hRPA3) subunits (Wobbe et al., 1987; Fairman and Stillman, 1988; Wold and Kelly, 1988). Genetic and biochemical analysis of RPA from various eukaryotes has revealed it to play critical roles during the initiation and elongation stages of chromosomal DNA replication (Brill and Stillman, 1989; Heyer et al., 1990; Adachi and Laemmli, 1994; Maniar et al., 1997). Use of the SV-40 model system indicates that during DNA replication initiation, hRPA binds and extends an 8-nt ssDNA element generated within a complex formed between the SV-40 large tumor antigen (T antigen) and the SV-40 origin of replication (*ori*) (Dean et al., 1987a; Deb and Tegtmeyer, 1987; Borowiec and Hurwitz, 1988), leading to release of the T antigen DNA helicase activity (Dean et al., 1987b; Wold et al., 1987; Iftode and Borowiec, 1997). During elongation, hRPA modulates the activity of the DNA polymerase α/DNA primase complex (Matsumoto et al., 1990; Collins and Kelly, 1991; Melendy and Stillman, 1993; Braun et al., 1997), and physically interacts with both the DNA polymerase α complex and T antigen (Dornreiter et al., 1992).

RPA is required for nucleotide excision repair and homologous DNA recombination (Coverley et al., 1991; Longhese et al., 1994; Firmenich et al., 1995; Smith and Rothstein, 1995), and interactions of hRPA with DNA repair and recombination factors have been described. In nucleotide excision repair, hRPA binds the XPA and XPG repair factors (He et al., 1995), and properly positions the XPG and ERCC1-XPF nucleases on the damaged DNA

Address correspondence to James A. Borowiec, Department of Biochemistry, New York University School of Medicine, 550 First Avenue, New York, NY 10016. Tel.: (212) 263-8453. Fax: (212) 263-8166. E-mail: james.borowiec@med.nyu.edu

[1]*Abbreviations used in this paper:* AMD, actinomycin D; BrdU, bromodeoxyuridine; EcoSSB, *E. coli* SSB; GST, glutathione *S*-transferase; hRPA, human replication protein A; *ori*, SV-40 origin of replication; pre-rRNA, precursor ribosomal RNA; RIP, RPA-interacting protein; RPA, replication protein A; SSB, single-stranded DNA-binding protein; ssDNA, single-stranded DNA; T antigen, SV-40 large tumor antigen.

molecule (de Laat et al., 1998). For homologous recombination and double-strand DNA break repair, hRPA can interact with the human Rad51 protein (Golub et al., 1998), and both human and *Saccharomyces cerevisiae* RPA (scRPA) have been demonstrated to interact with the cognate rad52 protein (Park et al., 1996; Hays et al., 1998). Other RPA-interacting proteins (RIPs) include p53 (Dutta et al., 1993; He et al., 1993; Li and Botchan, 1993), the DNA-dependent protein kinase (Brush et al., 1994; Pan et al., 1994), and the ataxia telangiectasia-mutated gene (ATM) protein kinase (Gately et al., 1998).

To identify novel pathways that regulate hRPA activities in DNA metabolism and cell growth, we used hRPA affinity chromatography to isolate novel RIPs. Here we report the identification of an interaction between hRPA and nucleolin, an abundant nuclear protein essential for the first step of precursor ribosomal RNA (pre-rRNA) processing. Nucleolin inhibits both *ori*-unwinding and SV-40 replication in vitro through a physical interaction with hRPA. Heat shock treatment of HeLa cells causes a dramatic redistribution of nucleolin from the nucleolus to the nucleoplasm, resulting in a great increase in nucleolin–hRPA complex formation. Thus, these data indicate that nucleolin has a second function distinct from its pre-rRNA processing role in modulating DNA metabolism in response to stress.

Materials and Methods

Preparation of Proteins

Recombinant hRPA was expressed in and purified from *Escherichia coli* as described previously (Henricksen et al., 1994; Iftode and Borowiec, 1998). The hRPA1ΔC442 and hRPA2/hRPA3 purified proteins (originally RPA70ΔC442 and RPA32·14 in Walther et al. [1999]) were a kind gift from Marc Wold (University of Iowa, Iowa City, IA). *E. coli* SSB (EcoSSB) was purchased from United States Biochemical. T antigen was purified from Sf9 insect cells infected with recombinant baculovirus as described previously (Borowiec et al., 1991).

The His_6-tagged hRPA3 (His-hRPA3) was overexpressed in *E. coli* from pCU232 (generously provided by Tom Kelly, Johns Hopkins University, Baltimore, MD) (Umbricht et al., 1993). After cell lysis, the insoluble His-hRPA3 was solubilized by resuspension in 50 mM Tris-HCl, pH 7.5, and 6 M urea. The material was clarified by centrifugation and quickly diluted into a 10-fold excess of 50 mM Tris-HCl, pH 7.5, 0.2 M NaCl, and 1 M 3(1-pyridinio)-1-propane sulfonate (Vuillard et al., 1995). The refolded protein was then purified on a Ni^{2+} column using standard procedures.

Nucleolin was isolated using a modification of the procedure described by Dickinson and Kohwi-Shigematsu (1995). In brief, a K562 cell extract (100 mg) was dialyzed against buffer Z (25 mM Hepes, pH 7.8, 12.5 mM $MgCl_2$, 1 mM DTT, 20% glycerol, and 0.1% NP-40) containing 100 mM KCl. The extract was loaded onto a 10-ml phosphocellulose column previously equilibrated with buffer Z containing 100 mM KCl. After extensive washing, nucleolin was eluted with 10 ml of buffer Z containing 0.5 M KCl. The presence of nucleolin was monitored by Western blot analysis and chemiluminescence detection (Boehringer). The eluate was loaded onto a 10-ml Affigel Blue column equilibrated with buffer Z containing 0.5 M KCl, and eluted with 10 ml of buffer Z containing 1.5 M sodium thiocyanate. After dialysis against buffer Z containing 100 mM KCl, the protein was loaded onto and eluted from a DNA affinity column containing double-stranded DNA molecules that include a high-affinity binding site for nucleolin (Dickinson and Kohwi-Shigematsu, 1995). The DNA construct was generously provided by Terumi Kohwi-Shigematsu (University of California, Berkeley, CA). The eluted nucleolin (~10 μg) was estimated to be >98% pure as judged by SDS-PAGE and silver staining.

Isolation and Identification of RIPs

Crude cytosolic extracts of HeLa cells (Cell Culture Center) prepared as described previously (Wobbe et al., 1987) were used to isolate the RIPs. The HeLa extract (~1 g) was first precleared of endogenous hRPA by passage through a 20-ml ssDNA-cellulose (Amersham Pharmacia Biotech) column equilibrated with buffer Y (20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.01% [vol/vol] NP-40, 1 mM DTT, 1 mM PMSF) containing 0.5 M NaCl. To remove general ssDNA-binding proteins from the hRPA-depleted extract, the ssDNA-cellulose flow-through was dialyzed against buffer Y containing 100 mM NaCl and passed through a 20-ml ssDNA-cellulose column similarly equilibrated.

The hRPA affinity column was prepared by loading 0.2 mg of recombinant hRPA onto a 2-ml ssDNA-cellulose column. High-affinity RIPs were then prepared by applying the 0.1 M NaCl flow-through from the second ssDNA-cellulose column (above) to the hRPA affinity column equilibrated with buffer Y containing 100 mM NaCl. After washing the column with 20 ml of buffer Y containing 100 mM NaCl, the column was sequentially eluted with 3 ml of buffer Y containing 0.3 and 0.5 M NaCl. The eluates were individually concentrated using a Centricon YM-10 centrifugal filter (Amicon), and separated by electrophoresis through an 8% SDS-PAGE gel (acrylamide:bisacrylamide, 29:1). After Coomassie blue staining, prominent bands were excised and sent for protein sequence analysis to the Protein/DNA Technology Center at Rockefeller University.

Immunoprecipitation and Immunoblotting

Immunoprecipitations were performed in 0.5 ml HeLa lysate using the IMMUNOcatcher system (CytoSignal). For interaction studies using purified proteins, reaction mixtures (20 μl) containing 20 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 0.01% NP-40, 50 mM KCl, and 250 ng of each protein were first incubated for 30 min at 4°C, and then immunoprecipitated. For immunoprecipitations from actinomycin D (AMD)-treated and heat-shocked cells, extracts were prepared using the same method as in Wobbe et al. (1987), except that 0.5% NP-40 was included in all buffers. Identification of precipitated proteins was achieved by Western blot analysis using chemiluminescence detection. Antibodies used in these procedures were generously provided by M. Wold (University of Iowa) and Suk-Hee Lee (Indiana University School of Medicine, Indianapolis, IN) (polyclonal antibodies directed against hRPA2 and hRPA3, respectively), Mark Kenny (Montefiore Medical Center, Bronx, NY) (mAbs directed against hRPA1 and hRPA2), and Harris Busch (Baylor College of Medicine, Houston, TX) (a mAb directed against nucleolin). The anti–glutathione *S*-transferase (GST) antibody was purchased from Amersham Pharmacia Biotech.

ori Denaturation and SV-40 DNA Replication In Vitro

The *ori*-containing plasmid used for in vitro replication assays contained a 90-bp SV-40 fragment (positions 5186 to 32) subcloned into the BamHI and XhoI sites of pBluescript SK+ phagemid (Stratagene). The DNA substrate used for *ori* denaturation was prepared by excising this same 90 bp, which was then labeled with ^{32}P as described previously (SenGupta and Borowiec, 1994). Standard *ori* DNA unwinding and SV-40 replication reactions were performed as described previously (Wobbe et al., 1985; SenGupta and Borowiec, 1994).

Chromosomal DNA Replication Assay

HeLa cells were grown in 96-well microtiter plates, and cellular DNA synthesis assayed using the Cell Proliferation ELISA kit (Boehringer). Cells were either incubated at 37°C without treatment, treated with AMD (final concentration 0.1 μg/ml) or cycloheximide (final concentration 50 μg/ml) for 1 h at 37°C, or incubated for 90 min at 44°C to heat shock cells. Cells were then incubated with 10 μM bromodeoxyuridine (BrdU) for 30 min at the identical temperature. Cells were washed, fixed, and assayed for BrdU incorporation colorimetrically as described in the manufacturer's instructions. The BrdU signal ($A_{450} - A_{690}$), detected using an ELISA reader, was the absorbance at 450 nm minus the background absorbance at 690 nm. The reported signal was also corrected by subtracting the ($A_{450} - A_{690}$) value found when cells were incubated without BrdU.

Immunofluorescence Microscopy

HeLa cells were grown on coverslips in preparation for epifluorescence microscopy. UV-treated cells were incubated for 2 h at 37°C after a single 30 J/m^2 treatment. AMD-treated cells were grown for 1 h at 37°C in the presence of 0.1 μg/ml AMD (Sigma). To induce heat shock, cells were grown for 90 min at 44°C. Cells were prepared for microscopy as de-

scribed in Dimitrova et al. (1999). Basically, cells were washed with cold cytoskeleton (CSK) buffer containing 0.3 M sucrose, and then incubated for 2 min in this same buffer containing 0.5% Triton X-100. After this extraction step, cells were washed carefully and incubated for 30 min in 4% (wt/vol) formaldehyde at room temperature. The primary antibody used was a mAb directed against nucleolin, whereas the secondary antibody was an FITC-conjugated donkey anti–mouse IgG (Jackson ImmunoResearch Laboratories). Epifluorescence microscopy was performed with a Zeiss Axiophot microscope.

Coverslips for confocal microscopy were prepared similarly. Cells were supplemented with 30 μg/ml BrdU (Sigma) for 10 min before fixation. The primary antibodies used were a rabbit anti-RPA2 polyclonal, a mouse anti-nucleolin monoclonal, and a rat monoclonal against BrdU. The secondary antibodies used were an anti–rabbit Cy5-conjugated donkey IgG, an anti–mouse Texas red–conjugated donkey IgG, and an anti–rat FITC-conjugated donkey IgG, respectively. Images were acquired as single scans on a Leica Confocal Microscope using TCS NT software. Images were further analyzed and colocalization determined using MetaMorph (Universal Imaging). Quantitation of colocalization was performed on various confocal field images, each containing ~10 cells. The threshold level for the signal was mathematically determined by the software program and results in each pixel being scored as positive or negative for the stain. The colocalization data was carefully examined testing a variety of different threshold parameters, etc., to verify that the determined values were representative of the actual degree of colocalization and not a result of the parameter settings. All digital microscopic images were prepared using Adobe Photoshop 4.0.

Results

Identification of Nucleolin as a RIP

An affinity chromatography–based technique was used for the isolation of RIPs (Fig. 1 A). We chose an ssDNA-cellulose matrix as the hRPA-binding platform because of the high affinity that hRPA displays towards ssDNA, and to generate a binding target that represents hRPA in its extended, DNA-bound form (Blackwell et al., 1996). To disrupt endogenous hRPA–RIP complexes and to preclear the HeLa lysate of hRPA and general ssDNA-binding proteins, the lysate was sequentially passed through ssDNA-cellulose matrices equilibrated to 500 and 100 mM NaCl. This protocol is expected to generate a lysate that contains RIPs that bind hRPA with moderate affinity. The flow-through from these columns (in 100 mM NaCl) was applied to a ssDNA-cellulose column bound with bacterially overexpressed hRPA, and then eluted sequentially with 300 and 500 mM NaCl.

When separated by SDS-PAGE, we observed ~5–10 bands from each salt elution. Apart from two major bands found in both fractions, each eluate yielded a generally different pattern of bands (Fig. 1 B). Subjecting the material to a second round of binding and elution yielded the same profiles, indicating that the procedure was not artifactually isolating highly abundant proteins that bound the matrix because of a mass action effect (data not shown). One conspicuous band eluting at 500 mM NaCl and migrating at 100 kD (Fig. 1 B, lanes 3 and 4) was excised and subjected to sequence analysis. The sequence of two internal peptides (peptide 1, GFGFVDFNSEEDAK; peptide 2, FXYVDFESAXDLEK) each showed a perfect match to human nucleolin. In addition, the 500 mM eluate was subjected to immunoblot analysis using an anti-nucleolin antibody and found to contain a cross-reacting 100-kD band (data not shown). Although it is possible that a protein with significant homology to nucleolin exists in human cells, a more likely explanation is that hRPA affinity chromatography generates nucleolin as a high-affinity RIP.

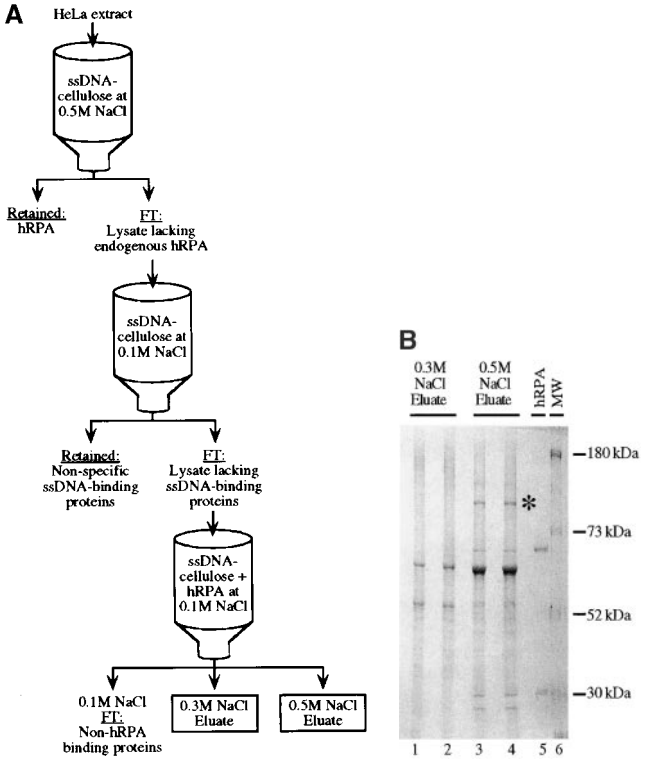

Figure 1. Identification of nucleolin as a RIP. (A) Scheme for the isolation of RIPs. A HeLa lysate was sequentially applied to individual ssDNA-cellulose columns equilibrated at 500 and 100 mM NaCl to generate a cellular extract lacking endogenous hRPA and other general SSBs. The flow-through from the second column was applied at 100 mM NaCl to a ssDNA-cellulose column preloaded with recombinant hRPA as described in Materials and Methods. The column was washed extensively and sequentially eluted with 300 and 500 mM NaCl. (B) Separation and visualization of isolated RIPs. The eluates from the hRPA affinity column were concentrated and separated by electrophoresis through an 8% SDS-PAGE gel and stained with Coomassie blue (lanes 1 and 2, 300 mM eluate; lanes 3 and 4, 500 mM eluate; each pair of lanes are duplicates). Also included were purified hRPA (lane 5) and molecular weight markers (lane 6). The 100-kD band (marked with an asterisk) was excised and identified as nucleolin by microsequencing analysis.

Nucleolin is a multifunctional protein with high abundance in the nucleolus (Ginisty et al., 1999; see below). Perhaps its best characterized function is its requirement in the first step of pre-rRNA processing in which the 5′ external transcribed spacer (ETS) is cleaved (Ginisty et al., 1998). Mutation of the genes encoding nucleolin homologues in *S. cerevisiae* (*NSR1*) and *Schizosaccharomyces pombe* (*gar2*$^+$) each results in a derangement of ribosome biogenesis and cellular growth (Kondo and Inouye, 1992; Lee et al., 1992; Gulli et al., 1995).

Characterization of the Nucleolin–hRPA Interaction

We examined if a nucleolin–hRPA complex can be detected in cellular lysates. Immunoprecipitation using an anti-hRPA3 antibody caused the coimmunoprecipitation of nucleolin from cellular extracts (Fig. 2 A, lanes 4 and 5).

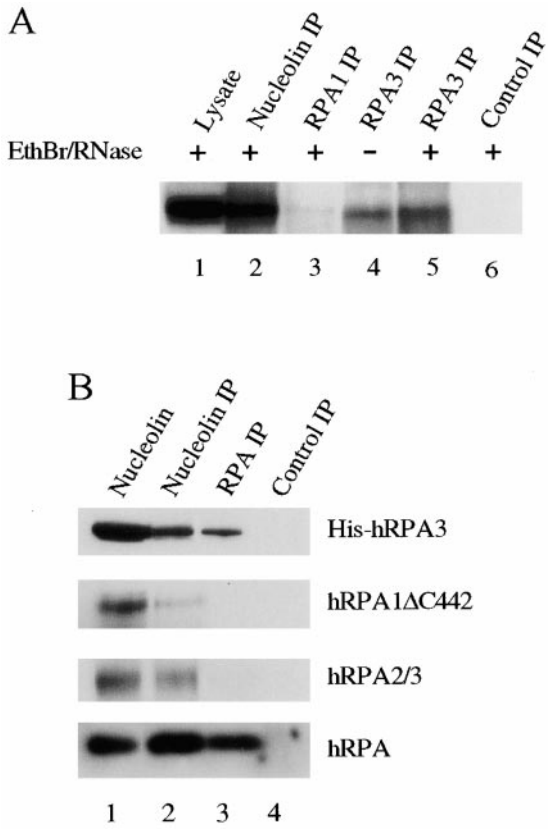

Figure 2. Nucleolin and hRPA physically interact via the hRPA3 subunit. (A) hRPA was immunoprecipitated from HeLa extracts using antibodies directed against the RPA1 (lane 3) or RPA3 (lanes 4 and 5) subunits. As controls, immunoprecipitations were performed using an anti-nucleolin (lane 2) or an anti-GST (lane 6) antibody. After SDS-PAGE of the immunoprecipitates, the separated material was subjected to immunoblotting analysis using anti-nucleolin antibodies. An aliquot of the HeLa lysate (40 μg) was electrophoresed in lane 1 as a positive control to indicate the position of nucleolin. RNase and ethidium bromide (EthBr) were added to all immunoprecipitation reactions except that shown in lane 4. (B) Purified nucleolin and His-hRPA3 (top panel), hRPA1ΔC442 (second panel), the hRPA2/3 subcomplex (third panel), or heterotrimeric hRPA (bottom panel) were incubated. After the incubation, reaction mixtures were immunoprecipitated using antibodies directed against nucleolin (lane 2, all panels), the hRPA3 subunit (lane 3, top and bottom panels), the hRPA1 subunit (lane 3, second panel), the hRPA2 subunit (lane 3, third panel), or GST (lane 4, all panels). The immunoprecipitates were separated by SDS-PAGE and visualized by immunoblotting analysis using anti-nucleolin antibodies. Purified nucleolin was electrophoresed in each lane 1 to provide a positive control.

Antibodies directed against the hRPA1 (Fig. 2 A, lane 3) or hRPA2 (data not shown) subunits were also successful at coimmunoprecipitating nucleolin, but the amount of nucleolin captured was ~10% of that found with the anti-hRPA3 antibody. A control antibody (against GST) failed to immunoprecipitate nucleolin (Fig. 2 A, lane 6). To ascertain that the interaction was direct and not mediated by a DNA or RNA bridge, extracts were pretreated with ethidium bromide and RNase (Lai and Herr, 1992). This treatment stimulated complex formation approximately twofold between hRPA and nucleolin (Fig. 2 A, compare lanes 4 and 5), demonstrating that the interaction is not dependent on the presence of nucleic acid. Control experiments also showed that an anti-nucleolin antibody coimmunoprecipitated hRPA, with all three hRPA subunits detectable (data not shown).

To determine if nucleolin directly interacts with hRPA, we examined the ability of purified hRPA or hRPA subcomplexes to bind nucleolin. Purified nucleolin was incubated with intact recombinant hRPA, the NH_2-terminal 441 amino acids of the hRPA1 subunit (which we term hRPA1ΔC442; originally RPA70ΔC442 in Walther et al. [1999]), a complex of the hRPA2 and hRPA3 subunits (which we term hRPA2/3; originally RPA32·14 in Walther et al., 1999), or a soluble peptide containing a His_6-tagged hRPA3 subunit (Umbricht et al., 1993).

Anti-hRPA antibodies failed to immunoprecipitate nucleolin in reactions containing hRPA1ΔC442 or the hRPA2/3 complex (middle two panels of Fig. 2 B, lane 3), yet succeeded in immunoprecipitating nucleolin when His-hRPA3 (Fig. 2 B, top panel, lane 3) or the heterotrimeric hRPA were used (Fig. 2 B, bottom panel, lane 3). In addition, human nucleolin was found to be retained when HeLa lysates were passed through a Ni^{2+} affinity column bound by the His-hRPA3 subunit (Kim, K., and J.A. Borowiec, data not shown). As the hRPA2/3 complex was not found to interact with nucleolin, it is possible that the nucleolin-interacting domain on hRPA3 is masked by hRPA2 in the subcomplex, thus preventing nucleolin binding. We conclude that hRPA and nucleolin can interact directly, and that this interaction is mediated through the small hRPA subunit.

To the best of our knowledge, this is the first characterized hRPA interaction that maps specifically to the hRPA3 subunit. Interestingly, immunofluorescence studies indicate the presence of hRPA3 in the nucleolus, apparently in the absence of the other two hRPA subunits (Murti et al., 1996; Dimitrova and Gilbert, 2000; Daniely, Y., and J.A. Borowiec, unpublished observations). Although it is possible that a separate interaction between nucleolin and a pool of free hRPA3 exists in vivo, further work is needed to conclusively demonstrate this possibility.

Nucleolin Inhibits hRPA-mediated ori Unwinding and DNA Replication

To determine the functional significance of the hRPA–nucleolin interaction, we examined whether nucleolin could influence various hRPA-mediated reactions. We first determined the effect of nucleolin on denaturation of *ori*, a subreaction of SV-40 DNA replication (Goetz et al., 1988; Wiekowski et al., 1988). In this reaction, the addition of T antigen, hRPA, and ATP to an *ori*-containing duplex DNA fragment results in denaturation of the DNA substrate (Fig. 3 A, lane 2). Significantly, addition of nucleolin (equimolar with hRPA) to the reaction nearly completely abolished *ori* denaturation by T antigen and hRPA (Fig. 3 A, lane 3). To rule out the possibility that the effect of nucleolin is indirect, we replaced hRPA by EcoSSB, which efficiently supports *ori* denaturation (Fig. 3 A, lane 4) (Kenny et al., 1989; Iftode and Borowiec, 1997). In the presence of EcoSSB, the addition of nucleolin only re-

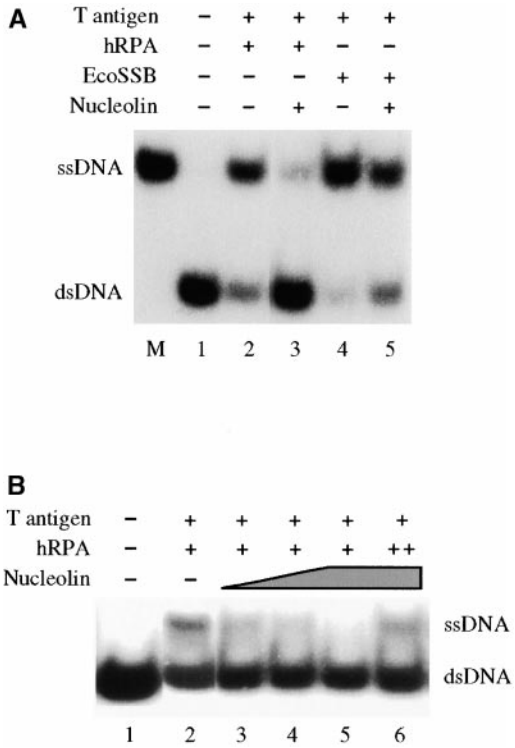

Figure 3. Nucleolin inhibits SV-40 *ori* unwinding through an interaction with hRPA. (A) A ^{32}P-labeled *ori*-containing DNA fragment (0.15 pmol; lane 1) was incubated in the presence of T antigen (1 μg; lanes 2–5), either hRPA (1 μg; lanes 2 and 3) or EcoSSB (1 μg; lanes 4 and 5), and nucleolin (1 μg; lanes 3 and 5) as described (SenGupta and Borowiec, 1994). Reaction products were separated by electrophoresis through an 8% nondenaturing gel and visualized by autoradiography. The position of the duplex DNA substrate and denatured product are indicated. Lane M shows the migration of the heat-denatured DNA fragment. (B) Increasing levels of nucleolin (50 ng, lane 3; 100 ng, lane 4; 250 ng, lanes 5 and 6) were incubated with T antigen (1 μg) and hRPA (250 ng, lanes 2–5; 500 ng, lane 6). Reaction products were detected as described in A.

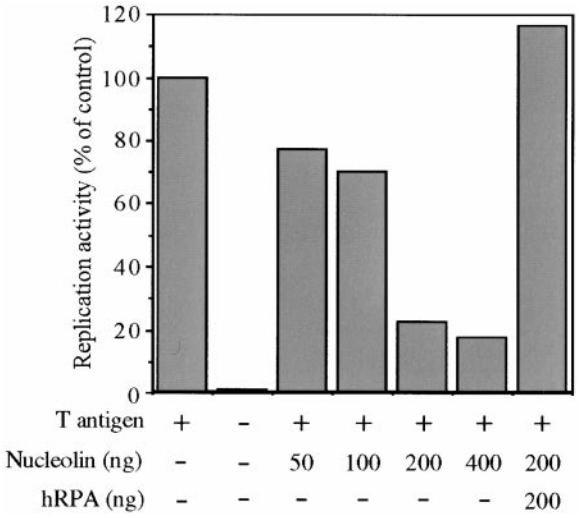

Figure 4. Nucleolin inhibits SV-40 DNA replication in vitro. An SV40 *ori*-containing plasmid (180 ng) was incubated with HeLa extract (350 μg), T antigen (750 ng), nucleolin (as indicated), and hRPA (as indicated) for 2 h at 37°C (Wobbe et al., 1985). Replication activity was determined by precipitating the reaction mixtures with trichloroacetic acid. The amount of ^{3}H in the precipitate was measured by scintillation counting. The relative replication efficiency of each reaction was defined as the percentage of that found in the control reaction containing T antigen but lacking nucleolin (34.2 pmol incorporated).

duced the amount of ssDNA generated by ~25% (Fig. 3 A, lane 5; data not shown), demonstrating that the inhibition of *ori* denaturation is primarily mediated through a specific nucleolin–hRPA interaction.

Because we found that nucleolin physically interacts with hRPA in solution, it is possible that nucleolin inhibits *ori* denaturation by sequestering hRPA in a nonactive state. If this model is correct, then the inhibitory effects of nucleolin should be overcome by the addition of more hRPA. To test this possibility, increasing levels of nucleolin were added to an *ori* denaturation reaction using hRPA (Fig. 3 B). Note that less hRPA was used for the control reaction (Fig. 3 B, lane 2; 250 ng) than in the corresponding reaction shown in Fig. 3 A, lane 2 (1 μg), to allow greater sensitivity to nucleolin inhibition, although causing a less robust DNA unwinding reaction. In the presence of 250 ng of nucleolin, the *ori* denaturation reaction was completely inhibited (Fig. 3 B, lane 5). Augmenting the reaction with 250 ng of additional hRPA was seen to nearly completely reverse the inhibitory effects of nucleolin (Fig. 3 B, lane 6). These data show that the formation of a nucleolin–hRPA complex prevents hRPA from productively interacting with the T antigen–*ori* complex during the initiation of DNA replication.

We tested the effect of nucleolin in the more complete SV-40 DNA replication reaction in vitro using crude HeLa lysates (Fig. 4). As was found for *ori* denaturation, titration of increasing levels of nucleolin greatly inhibited the replication reaction, with ~80% less DNA synthesis seen in the presence of 200 ng of exogenous nucleolin. Supplementing this reaction with 200 ng of recombinant hRPA restored replication to a level slightly above control levels. The addition of hRPA to a reaction lacking exogenous nucleolin had little if any effect on DNA synthesis relative to the control reaction (data not shown). Nucleolin thus has the ability to inhibit the initiation of DNA replication through a direct interaction with hRPA.

Cellular Heat Shock Relocalizes Nucleolin from the Nucleolus to the Nucleoplasm

We reasoned that the inhibitory effects of nucleolin on hRPA activity may reflect a mechanism that is induced under particular stress conditions. In this scenario, certain cellular trauma may induce changes in nucleolin localization, leading to increased nucleolin–hRPA complex formation, which facilitates the repression of chromosomal DNA replication. To explore this hypothesis, we examined the localization of nucleolin under various stress conditions. In control HeLa cells, nucleolin is almost completely localized to the nucleolus with only faint nucleoplasmic

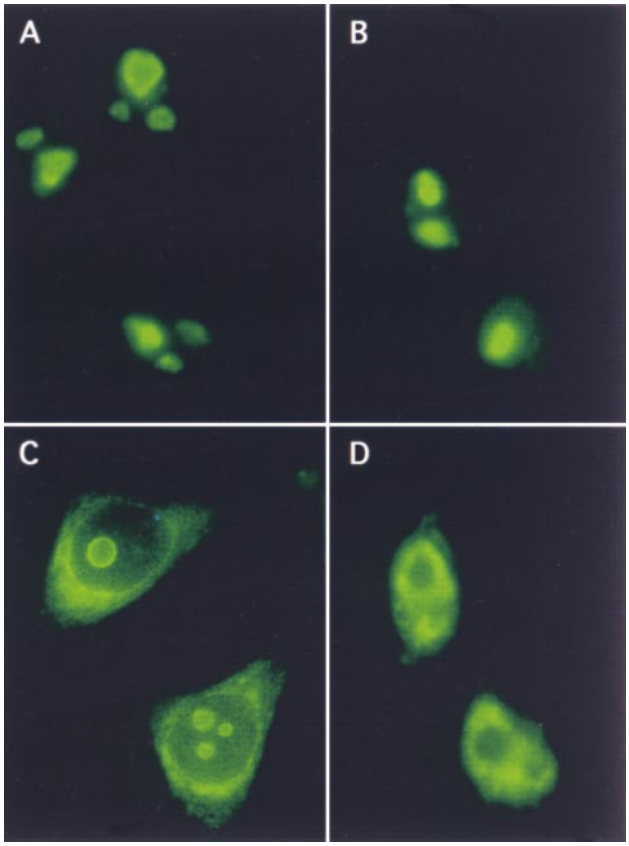

Figure 5. Nucleolin delocalizes from the nucleolus upon heat shock and stably associates with nucleoplasmic structures. HeLa cells were either untreated (A) or subjected to UV irradiation (30 J/m^2; B) treatment with AMD (0.1 μg/ml; C) or heat shock (90 min at 44°C; D). Cells were then fixed by treatment with 4% (wt/vol) formaldehyde for 30 min at room temperature and then stained for nucleolin. Nucleolin localization was determined by epifluorescence microscopy using a Zeiss Axiophot.

staining (Fig. 5 A). Irradiation of cells with UV light had no effect on nucleolin localization (Fig. 5 B), although this treatment did cause increased nucleoplasmic staining of hRPA (data not shown). In contrast, shutdown of RNA polymerase I transcription by treatment with AMD (0.1 μg/ml) induced a predominantly nucleolar-to-cytoplasmic change in nucleolin localization (Fig. 5 C), although residual nucleolar staining was observed. Most significantly, heat treatment of cells (90 min at 44°C) caused a dramatic redistribution of nucleolin from the nucleolus to the nucleoplasm (Fig. 5 D). Under these conditions, the nucleolus was greatly depleted of nucleolin (dark areas in the nucleus), and nucleoplasmic staining of nucleolin was nonuniform and granular in appearance.

A time course of this phenomena indicated that nucleolin relocalization began after ~30 min at the elevated temperature. After 180 min at 44°C, cells began to detach from the plate (data not shown). The recovery from a 90-min heat shock at 44°C was also examined. After heat treatment, the cells were shifted back to 37°C for 24 h. A great majority of cells (>90%) was viable and nucleolin was again found to be localized to the nucleolus (data not shown). A more complete examination of the recovery phase is currently under study.

Table I. Effect on Chromosomal DNA Replication of Heat Shock and Metabolic Inhibitors

Condition	Absorbance ($A_{450} - A_{690}$)	Relative BrdU incorporation
		%
Control	0.122 ± 0.019	100
Heat shock	0.016 ± 0.004	13.1
AMD	0.112 ± 0.018	91.8
Cycloheximide	0.147 ± 0.026	120

HeLa cells were grown in 96-well microtiter plates and subjected to heat shock (1.5 h at 44°C) or treatment with either AMD (0.1 μg/ml for 1 h at 37°C) or cycloheximide (50 μg/ml for 1 h at 37°C). Cells were then incubated with 10 μM BrdU for an additional 30 min at the same temperature. The amount of BrdU incorporated was determined colorimetrically as described in Materials and Methods. Reported results are the average of at least three separate experiments.

Importantly, heat shock has been shown previously to strongly repress mammalian chromosomal DNA replication (Roti Roti et al., 1997; see Table I), with a recent study finding that HeLa DNA replication is inhibited ~70% after 15 min at 44°C (Wang et al., 1998). The repressive effects on DNA replication have been observed to occur at multiple steps, including a strong inhibition of replication origin firing, a two- to fivefold reduction in the rate of chain elongation, and defective processing of replication intermediates to fully ligated products (Wong and Dewey, 1982; Warters and Stone, 1983, 1984; Warters, 1988; Warters and Lyons, 1990). Depending on the incubation temperature employed, heat shock increases the transit time through the G1 and S phases of the cell cycle (Kal et al., 1975; Schlag and Lücke-Huhle, 1976; Sapareto et al., 1978). Predictably, cell cycle position greatly affects the heat sensitivity of mammalian cells, with G1 and G2 cells found more resistant to hyperthermic temperatures than cells in S phase (Dewey et al., 1978). Recent biochemical investigation suggests that the inhibitory effects of heat shock on DNA replication initiation are mediated through RPA (Wang et al., 1998).

Because AMD can also cause nucleolin relocalization (albeit to the cytoplasm), we considered the possibility that repression of DNA replication is an indirect effect resulting from the inhibition of rRNA transcription or protein translation, rather than a direct consequence of nucleolin acting in the nucleoplasm. We tested the effect on chromosomal DNA replication of heat shock (90 min at 44°C), inhibition of RNA polymerase I transcription by treatment with AMD (0.1 μg/ml), or the shutdown of protein translation by treatment with cycloheximide (50 μg/ml). Cells were treated with AMD and cycloheximide for 1 h rather than 90 min, as these conditions completely inhibit RNA polymerase I transcription and protein translation, respectively. As measured by the ability of cells to incorporate BrdU (Table I), heat shock inhibited DNA synthesis to a level ~15% of that found in control cells, similar to results found by others (e.g., Wang et al., 1998). In contrast, AMD or cycloheximide did not significantly affect cellular BrdU incorporation. These data indicate that the rapid inhibition of chromosomal DNA synthesis is not merely a consequence of a block to rRNA production or protein translation.

Heat Shock Induces Nucleolin–hRPA Complex Formation

The movement of nucleolin to the nucleoplasm after heat shock predicts a concomitant increase in the level of hRPA–nucleolin complex formation. To examine this possibility, lysates were prepared from control cells or cells that were subjected to heat shock or treatment with AMD. From these lysates, hRPA was immunoprecipitated and the immunoprecipitates examined by Western blot analysis for the presence of nucleolin (Fig. 6). A low but significant amount of nucleolin coimmunoprecipitated with hRPA in control cells (Fig. 6, top panel, lane 1). In cells treated with AMD, the amount of nucleolin precipitating with hRPA increased approximately fourfold. We did not observe any change in the subcellular localization of hRPA after AMD treatment (data not shown), suggesting that nucleolin transit through the nucleoplasm binds a fraction of the hRPA pool, but this complex remains soluble and is not retained after our epifluorescence fixation procedure.

Lysates from heat-shocked cells contained ~10-fold increased levels of hRPA–nucleolin complex formation over lysates from control cells (Fig. 6, top panel, lane 3). Control blots showed that heat shock or AMD treatment did not affect the amount of hRPA precipitated by the anti-hRPA2 antibodies (Fig. 6, middle panel), or the amount of nucleolin in the lysate (Fig. 6, bottom panel). We therefore find that heat shock causes nucleolin to relocalize to the nucleoplasm, and this is accompanied by an increased level of nucleolin–hRPA complex.

Nucleoplasmic Colocalization of Nucleolin and hRPA after Heat Shock

We wished to determine if the nucleolin–hRPA complexes induced after heat shock were observable in discrete nucleoplasmic sites. Moreover, if such nucleoplasmic complexes could be detected, we wished to examine their location relative to sites of DNA synthesis. These questions were addressed quantitatively using confocal microscopy (Fig. 7). Control or heat-shocked (44°C for 1.5 h) HeLa cells were incubated with BrdU for the final 10 min before fixation and then stained using anti-nucleolin, anti-hRPA2, and anti-BrdU antibodies. Standard immunostaining procedures, which do not extract freely soluble RPA, yield uniform nuclear staining of hRPA (e.g., Kenny et al., 1990). We therefore included a prefixation extraction step (as described by Dimitrova et al. [1999]) that allows visualization of hRPA bound to chromosomal replication factories and other nuclear structures.

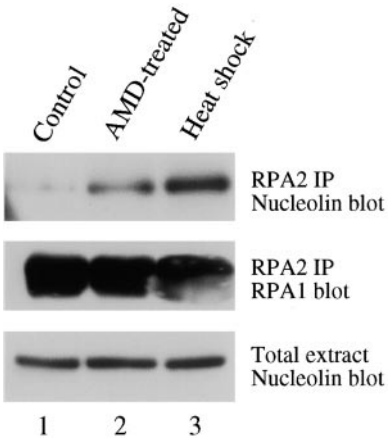

Figure 6. Heat shock greatly increases nucleolin–hRPA complex formation. hRPA was immunoprecipitated from control HeLa cell extracts, or extracts from cells treated with AMD (0.1 μg/ml), or subjected to heat shock (lanes 1, 2, and 3, respectively, upper and middle panels) using antibodies directed against the hRPA2 (upper and middle panels) subunit. After SDS-PAGE of the immunoprecipitates, the separated material was subjected to immunoblotting analysis using anti-nucleolin antibodies (upper panel) or anti-RPA1 antibodies (middle panel). Lysates (20 μg) prepared from control, AMD-treated, and heat-shocked HeLa cells (lanes 1, 2, and 3, respectively, lower panel) were subjected to immunoblotting analysis using anti-nucleolin antibodies to monitor the amount and appearance of nucleolin in the extracts.

As seen in a representative control cell, nucleolin was nearly completely localized to the nucleolus (Fig. 7 A), whereas general nuclear staining of hRPA was observed (Fig. 7 B). Note that the nucleolin signal is found around rather than within the nucleolus. Although nucleolin has been detected preferentially at the nucleolar dense fibrillar component and less significantly at the fibrillar centers and granular component (e.g., Escande-Geraud et al., 1985), we postulate that the extraction procedure selectively removes nucleolin from internal regions of the nucleolus. Such perinucleolar staining has been observed previously by others (Welsh et al., 1999). hRPA was localized in a punctate pattern in a significant fraction of HeLa cells (i.e., those in S phase), whereas others had a minimal hRPA signal. The hRPA-positive cells were found almost invariably to also have a significant BrdU signal (e.g., Fig. 7 C) displayed in a granular pattern. Pairwise combinations of the staining patterns indicate that the hRPA and BrdU signals are nearly coincident (Fig. 7 E). The degree of colocalization between hRPA, nucleolin, and sites of BrdU incorporation in control cells was quantified from confocal field images of cells (Table II). Over 75% of the hRPA signal was found to colocalize with the sites of DNA synthesis. As would be anticipated from the composite images, a much smaller fraction of the hRPA or BrdU signal overlapped with that of nucleolin (16.0 and 19.4%, respectively).

Heat shock caused nucleolin to relocalize from the nucleolus to the nucleoplasm (Fig. 7 G), as found above. hRPA localization was relatively unaffected under these conditions (Fig. 7 H). Overlap of the two images indicate that nucleolin and hRPA show extensive colocalization (Fig. 7 J, sites of purple color). Although the overlap was widespread, hRPA was slightly overrepresented relative to nucleolin in the nuclear periphery. Quantitation of field images demonstrated that the fraction of hRPA signal that overlaps with nucleolin increases significantly after heat shock, from 16.0 to 61.2% (Table II). This is not merely a result of two widely dispersed signals giving artifactually coincident localization. As found below, only a small fraction of the BrdU signal colocalized with the nucleolin signal after heat shock (from 19 to 25%), even though both signals were highly abundant in the nucleoplasm. These data indicate that the induced nucleolin–hRPA complex that is formed after heat shock localizes in discrete nucleoplasmic sites.

A significant fraction of heat-shocked cells was found to incorporate BrdU, although the BrdU signal was significantly reduced compared with control cells (data not shown; see Table I). In these cells, punctate sites of nucle-

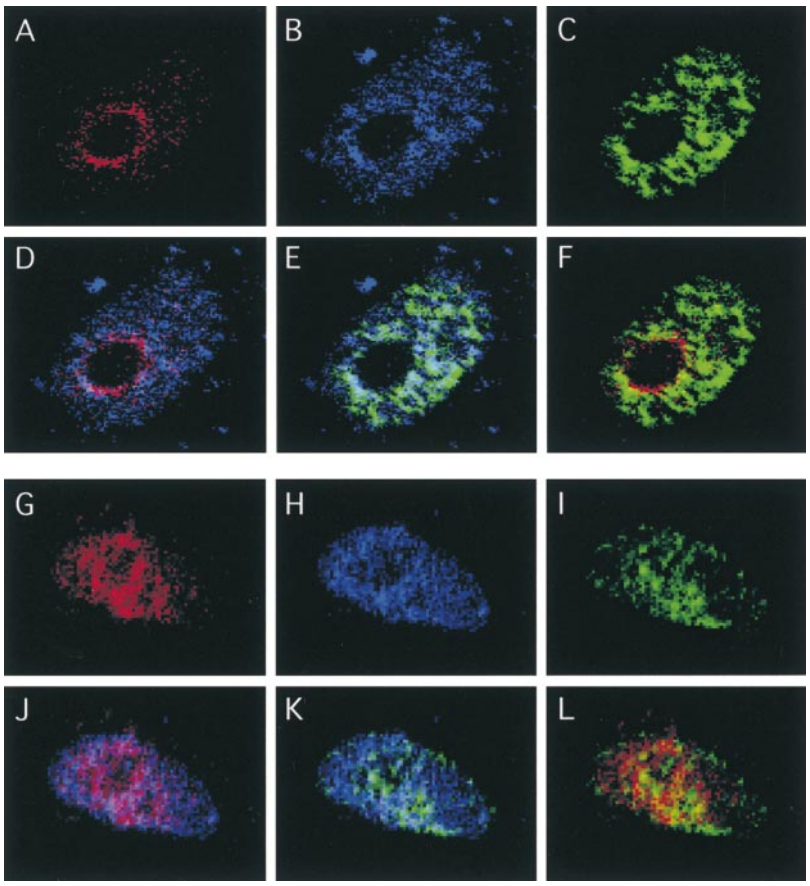

Figure 7. Confocal imaging of the effect of heat shock on the nucleoplasmic colocalization of nucleolin, hRPA, and sites of BrdU incorporation. Images were acquired either from control HeLa cells (A–F) or cells subjected to heat shock (90 min at 44°C, G–L). Cells were supplemented with BrdU (30 μg/ml) for the final 10 min of the incubation before fixation. After fixation, cells were stained with anti-nucleolin (A and G), anti-hRPA2 (B and H), or anti-BrdU (C and I) antibodies. Use of the anti-hRPA2 antibody reveals the localization of heterotrimeric hRPA. Composite images are shown, combining the staining pattern for hRPA and nucleolin (D and J), hRPA and BrdU (E and K), and nucleolin and BrdU (F and L). Note that the BrdU signal in I was digitally enhanced relative to the signal in C to allow visualization of the weaker BrdU signal in heat-shocked cells (e.g., see Table I). The degree of colocalization of each signal was quantitated and reported in Table II, in which digital enhancement was not employed. Cells were prepared similarly as for epifluorescent microscopy, as described in the Fig. 5 legend and Materials and Methods. Images were captured on a Leica Confocal Microscope.

otide incorporation are clearly seen (Fig. 7 I). Visual inspection of the composite nucleolin–BrdU images (Fig. 7 L; data not shown) indicate that the areas of strong nucleolin staining were generally those with low levels of BrdU incorporation, and conversely, sites of intense BrdU incorporation showed relatively low nucleolin staining. This observation was confirmed by quantitation of the confocal data, which demonstrated that only ~25% of the BrdU signal overlapped with nucleolin (Table II). These data reveal that after heat shock, nucleolin only poorly colocalizes with sites of ongoing DNA synthesis. The fraction of hRPA signal that colocalized with BrdU after heat shock decreased from 78 to 44%, suggesting that nucleolin relocalization to the nucleoplasm after heat shock prevents a sizable fraction of the hRPA pool from participating in DNA synthetic events.

Table II. Quantitation of the Degree of Colocalization of Nucleolin, hRPA, and Sites of BrdU Incorporation in Control and Heat-shocked Cells

Signal 1/signal 2	Control	Heat shock
	%	%
BrdU/nucleolin	19.4 ± 1.1	24.8 ± 0.3
hRPA/nucleolin	16.0 ± 2.5	61.2 ± 2.4
hRPA/BrdU	78.1 ± 3.3	44.5 ± 3.9

Confocal field images were carefully analyzed using MetaMorph image processing software to quantitate co-localization. Co-localization is expressed as the percent positive signal (threshold level for each signal is mathematically determined by the MetaMorph program) which coincides with a positive signal in a corresponding image. For each signal 1/signal 2, the percent of signal 1 that co-localizes with signal 2 is provided. Data reported is a result of at least two separate measurements.

Discussion

Mammalian heat shock has multiple effects on DNA metabolism, including aberrant processing of DNA replication intermediates, a reduction in the rate of replication fork movement, and premature chromosome condensation, leading to cell death (Wong and Dewey, 1982; Warters and Stone, 1983, 1984; Warters, 1988; Warters and Lyons, 1990; Roti Roti et al., 1997). Compared with cells in G1 phase, S phase cells have decreased viability after heat shock, likely a result of replicative errors causing damaged DNA molecules to accrue at hyperthermic temperatures (Bhuyan et al., 1977; Dewey et al., 1978). Although heat shock does not cause an obvious G1/S block, it does lengthen the G1 phase and represses chromosomal DNA replication in S phase, most notably at the levels of replication initiation and the processing of replication intermediates (Kal et al., 1975; Schlag and Lücke-Huhle, 1976; Sapareto et al., 1978). Although the general inhibitory effects of hyperthermia on DNA replication are clear, the underlying molecular mechanisms are very poorly understood. Our data indicate that one critical element of this regulatory response is the relocation of nucleolin from the nucleolus to the nucleoplasm, where it binds hRPA and prevents hRPA from participating in the initiation of chromosomal DNA replication.

We demonstrated that nucleolin–hRPA complex formation significantly inhibited the initiation of DNA replication using the SV-40 system. We also showed that heat shock induced a 10-fold increase in nucleolin–hRPA complex formation, and these complexes appear to be localized at particular nucleoplasmic sites. Under heat shock conditions, nucleolin was generally excluded from sites of DNA synthesis. Although these data suggest that nucleolin is directly involved in the repression of chromosomal replication after heat shock, future studies are required to more fully explore the role of nucleolin in this process. Clearly, nucleolin relocalization after heat shock occurs with somewhat slower kinetics ($\sim$30 min; data not shown) than the inhibition of chromosomal DNA synthesis (10–15 min) (Wang et al., 1998). Because nucleolin does not inhibit hRPA from binding ssDNA (data not shown), our data indicate that nucleolin selectively inhibits hRPA from acting during replication initiation rather than elongation. We therefore postulate that the induction of nucleolin–hRPA complex formation is a key element of the repression of chromosomal origin firing after heat shock, but may not be involved to a significant degree in the inhibition of DNA synthesis at previously generated DNA replication forks. It is also possible that the nucleolin–hRPA complex induced upon heat shock has functions unrelated to its role in DNA replication, perhaps acting as a factor that signals cell stress. Intriguingly, nucleolin has recently been observed to relocalize to the nucleoplasm in response to the DNA-damaging agent mitomycin C, suggesting that nucleolin relocalization is a more general stress phenomena not specific to heat shock (David-Pfeuty, 1999).

Although over a dozen factors have been demonstrated to directly interact with RPA, few of these interactions have any known regulatory effects on RPA activity (Iftode et al., 1999). When the interaction has been found to have functional consequences, invariably it is the activity of the RPA-interacting factor that is modulated (e.g., DNA polymerase α/DNA primase) (Braun et al., 1997). The inhibitory effects of nucleolin on the productive interaction of hRPA with the SV-40 replication initiation complex thus represents a rare example of a factor that can regulate RPA function.

Nucleolin has only minor effects on the ssDNA-binding activity of hRPA (data not shown), indicating that its repressive effect on replication initiation is not caused by nucleolin directly blocking the primary ssDNA-binding domain of hRPA. More likely, the bound nucleolin sterically prevents hRPA from close approach to the ssDNA bubble within the T antigen–*ori* complex (Borowiec and Hurwitz, 1988; Parsons et al., 1990). Alternatively, nucleolin may alter the conformation of hRPA so as to prevent cooperative interactions with a second hRPA molecule, proposed to be critical for hRPA binding to and further unwinding of the ssDNA bubble within *ori* (Iftode and Borowiec, 1997). Because nucleolin can bind the nuclear localization signal of T antigen (Xue et al., 1993), it is conceivable that a secondary repressive effect may be mediated by a nucleolin–T antigen interaction. However, this is clearly not the major inhibitory pathway of nucleolin, because increasing levels of hRPA can overcome the repression of replication initiation by nucleolin, and nucleolin only weakly inhibits an *ori* denaturation reaction in which hRPA is replaced by EcoSSB.

Cells subjected to heat shock or treatment with AMD each show nucleolin redistribution, although the destined cellular compartment was the nucleoplasm or cytoplasm, respectively. Both heat shock and AMD treatment cause a significant decrease in the level of newly synthesized rRNA (Bell et al., 1988, Sadis et al., 1988), suggesting that loss of its pre-rRNA binding substrate is the common event that causes nucleolin release from the nucleolus. It is currently unclear why the two treatments result in nucleolin relocalization to different cellular compartments. Because nucleolin is known to be phosphorylated in a cell cycle– or growth factor–dependent manner by various kinases, including Cdc2, casein kinase II, and protein kinase C ζ (e.g., Ginisty et al., 1999), the posttranslational modification state of nucleolin may govern its redistribution to the nucleoplasm or cytoplasm.

After heat shock, we observed a significant nucleoplasmic signal for nucleolin even after an immunofluorescence fixation procedure that extracts free hRPA (Fig. 7). Nucleolin is therefore likely bound to a macromolecular structure that increases its retention in the nucleoplasm. The nucleoplasmic retention of nucleolin correlates with the results of past studies showing that heat shock enhances aggregation of proteins on the nuclear matrix (Roti Roti et al., 1997). Because of the ability of nucleolin to bind matrix attachment region (MAR) elements on chromosomal DNA (Dickinson and Kohwi-Shigematsu, 1995), it is possible that the nucleoplasmic nucleolin is chromatin-associated. hRPA does not appear to be a required factor in the nucleoplasmic localization of nucleolin, because in non–S phase cells, hRPA staining is insignificant, yet heat shock still induces nucleolin relocation to granular structures in the nucleoplasm (data not shown).

The identification of nucleolin as a RIP reveals a possible nexus between ribosome biogenesis and DNA metabolism. Nucleolin is required for the first step of pre-rRNA processing in which the 5′ external transcribed spacer undergoes an endonucleolytic cleavage (Miller and Sollner-Webb, 1981; Ginisty et al., 1998, 1999). In addition, mutation of the *S. cerevisiae NSR1* or *S. pombe gar2*$^+$ genes (that encode nucleolin homologues) has each been found to cause a derangement of rRNA processing. In certain prokaryotes such as *E. coli*, it is known that the level of key enzymes involved in macromolecular synthesis (e.g., DNA primase [dnaG], the ribosomal protein S21 [rpsU], and the RNA polymerase σ subunit [rpoD]) are coordinately regulated by expression from the macromolecular synthesis operon (Versalovic et al., 1993). In eukaryotes, it is unclear whether communication exists between the protein translation and chromosomal replication pathways. However, our finding of a functional interaction between hRPA and nucleolin demonstrates that these pathways are coupled, and cross-talk between these pathways can occur.

Recent studies of DNA replication using a variety of genetic and biochemical systems has revealed that eukaryotic DNA replication is subject to a variety of regulatory controls. In addition to the sophisticated levels of regulation that control the ability of an origin to initiate during a normal S phase (e.g., Leatherwood, 1998), chromosomal DNA replication is also subject to reversible arrest after

UV or ionizing irradiation, treatment with DNA-damaging agents, or heat shock (Friedberg et al., 1995; Roti Roti et al., 1997). For both ionizing and UV irradiation, it has been suggested that RPA is a key target for the inhibitory response (Liu and Weaver, 1993; Carty et al., 1994). Because we find that the repressive effects of heat shock on DNA replication can be mediated through RPA, this protein is emerging as a common target for cellular mechanisms that arrest the initiation of DNA replication after insult.

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