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Regulation of MDM2 Activity by Nucleolin

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A key antagonist of the p53 tumor suppressor is human MDM2 (Hdm2). We examined the significance of a recently identified complex between Hdm2 and nucleolin, a c-Myc-induced gene product with defined roles in ribosomal RNA processing and the inhibition of chromosomal DNA replication following stress. Changes in the level of nucleolin protein in unstressed cells cause parallel changes in the amount of p53 protein. Alterations in p53 levels arise from nucleolin binding to the p53-antagonist Hdm2, resulting in the inhibition of both p53 ubiquitination and Hdm2 auto-ubiquitination. Unexpectedly, we find that nucleolin also reduces Hdm2 protein levels, demonstrating that nucleolin inhibits Hdm2 using multiple mechanisms. Increases in nucleolin levels in unstressed cells led to higher expression of p21cip1/waf1, a reduced rate of cellular proliferation, and an increase in apoptosis. Thus, nucleolin has a number of properties in common with the tumor suppressor ARF. We propose that nucleolin, like ARF, responds to hyper-proliferative signals by up-regulation of p53 through Hdm2 inhibition. These findings have important implications on the progression of breast cells to cancer, and have the potential to provide new therapeutic routes to treat breast cancers.

MDM2, HDM2, nucleolin, P53, estrogen receptor alpha, ubiquitination
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INTRODUCTION

The human MDM2 (Hdm2) protein is overexpressed in one-third of benign breast lesions and two-thirds of malignant lesions. As Hdm2 normally causes rapid p53 turnover and reduces p53 transactivation, heightened Hdm2 levels will therefore inhibit p53-mediated cell-cycle arrest and apoptosis. We have found a novel interaction between Hdm2 and nucleolin, an abundant nucleolar protein, and this interaction apparently inhibits Hdm2 activity. The hypothesis to be tested in our studies is that nucleolin normally serves to inhibit Hdm2 activity in vivo. Increases in nucleolin protein levels will therefore inhibit Hdm2 ubiquitination activity, p53 nuclear exclusion, and ERα activity. The objective of the proposed work is to characterize the nucleolin-Hdm2 interaction and to understand the modulation of Hdm2 activities by nucleolin. The specific aims of the project are: (1) To identify the domains on both nucleolin and Hdm2 necessary for interaction, (2) to examine the effect of nucleolin on the ubiquitin ligase activity of Hdm2 for itself and for p53, (3) to examine the effect of nucleolin on the Hdm2-mediated nuclear exclusion of p53, and on Hdm2 localization, and (4) to characterize the effect of nucleolin on ERα activity and Hdm2-ERα complex formation in vivo.

BODY

Over the past three years, we have made significant progress analyzing the effect of nucleolin on the Hdm2 ubiquitin ligase activity. The key experimental findings are described below:

Tasks 1 and 2

• **Identification of Hdm2 and nucleolin domains required for nucleolin-Hdm2 complex formation.** We have generated numerous expression constructs for nucleolin and Hdm2. Using a Far Western approach, we observed that Hdm2 associated most strongly with a nucleolin construct containing the RBD and GAR domains, and more weakly to only the GAR domain. No binding was detected to the N-terminal domain (Fig. 1). These data indicate that nucleolin has highest affinity interactions with the central RBD domain, and a weaker interaction with the GAR domain.

• **Differential effect of nucleolin mutants on p53 stability.** We have tested various nucleolin fragments to examine the effect on p53 levels. Along with a full-length nucleolin control (NCL-FL), we also employed a construct that contains the N-terminal portion of nucleolin including the four RNA-binding domains (NCL-RBDs), and the C-terminal GAR domain (NCL-GAR). Interestingly, the two nucleolin mutants had opposite effects (Fig. 2). The RBD construct was found to cause elevated p53 levels compared to similar concentrations of the transfected full-length construct. The GAR domain actually appeared to depress the amount of p53 compared to wild-type nucleolin.

![Figure 1. Hdm2 binds to the nucleolin RBD and GAR domains.](image-url)
Combined with our binding studies (above), these data indicate that nucleolin has multiple effects on Hdm2 activity via distinct domains. That is, binding of the nucleolin RBD to Hdm2 has strong stabilizing effects on p53. In contrast, the GAR domain alone destabilizes p53. In the context of the complete molecule, an overall stabilization of p53 is noted. These data suggest that changes in the conformation of nucleolin (which we find occurs as a result of nucleolin phosphorylation; data not shown) could modulate the ability of nucleolin to control Hdm2 activity.

**Task 3**

- **Nucleolin over-expression increases p53 protein levels.** We characterized the effect of nucleolin on p53 protein levels in cells that normally express wild-type p53 (U2-OS, HCT116-wt) or in p53-null cell lines (H1299 or HCT116-ko) transfected with wild-type p53. We employed tagged-nucleolin constructs that have properties that are identical to those of endogenous nucleolin, including intracellular localization and ability to associate with RPA following genotoxic stress (1). Transfection of U2-OS cells with increasing levels of a plasmid expressing GFP-tagged nucleolin (GFPNu) was found to raise p53 levels nearly 4-fold relative to mock-transfected cells and nearly to the level of p53 found in cells treated with proteasome inhibitor MG132 (Fig. 3A). Transient or stable expression of nucleolin also increased the level of p53 in HCT116-wt cells (Fig. 3B) and in other cell lines (data not shown).

- **Nucleolin does not cause p53 activation in non-stressed cells.** Although we invariably over-express nucleolin only a maximum 2-fold over basal levels, it is possible that even a slight increase in nucleolin expression results in genotoxic stress, leading to p53 activation and stabilization. A hallmark of human p53 activation is phosphorylation of Ser15 by members of the PIKK family. Testing the status of Ser15, nucleolin increased the total p53 level by twofold, and the level of pSer15-p53 remained unaltered (Fig. 4; lanes 1 and 3; see also darker exposure). A parallel increase in endogenous p21cip1/waf1 protein levels was also observed (additional p53-downstream targets are examined in more detail below). In contrast, when cells transfected with GFPc (empty vector) or GFPNu were treated with the DNA-damaging agent camptothecin (CPT), similar increases in
both the total and pSer15-p53 were noted (lanes 2 and 4). These data indicate that nucleolin increases p53 levels in a pathway, at least in part, distinct from that utilized by cells undergoing genotoxic stress.

- **Down-modulation of nucleolin reduces p53 levels.** To more clearly show that nucleolin causes coordinate changes in the level of p53, we employed two different nucleolin-specific short-interfering RNA (siRNA) molecules, siNu1 and siNu2 to down-modulate nucleolin levels. Twenty-four hours post-transfection, both siNu1 and siNu2 molecules reduced the amount of nucleolin protein to 20% and 40%, respectively, of that observed in cells treated with a control siRNA molecule directed against luciferase (siLuc) (Fig. 5A; compare lanes 2 and 5 with lane 1). Control experiments found that the level of nucleolin in untransfected cells was similar to that detected in siLuc-treated cells (compare lanes 1 and 8). Reductions in the amount of p53 comparable to that of nucleolin were observed when p53 was examined by Western (Fig. 5A). Similar effects were seen by immunofluorescence microscopy (Fig. 5B). Using either siRNA molecule, cells with reduced nucleolin staining were also found to be deficient in p53. Overall, we find that alterations in the level of nucleolin cause parallel changes in the amount of p53 protein.

- **Nucleolin regulates the half-life of p53.** Because p53 levels are primarily governed through the regulation of p53 stability, we examined the influence of nucleolin on the p53 protein half-life. Following transfection of HCT116-wt cells with either GFPNu or GFPc expression vectors, cells were treated with cycloheximide and then harvested at various times post-treatment. Probing the lysates for p53 protein levels indicated a longer p53 half-life in cells expressing GFP-nucleolin (Fig. 6A). Densitometric analysis of the p53 levels, corrected for the level of β-actin in the same sample, confirmed this result (Fig. 6B). Our data indicate that heightened nucleolin expression results in significant stabilization of the p53 protein.

- **Nucleolin modulation of p53 levels is mediated by Hdm2.** The primary pathway of p53 turnover involves ubiquitination by Hdm2 and subsequent proteasomal degradation. Because our data suggest that nucleolin might interfere with this pathway, we examined...
the effect of Hdm2 on the ability of nucleolin to modulate p53 levels. We observed that nucleolin had a more pronounced effect on p53 levels in U2-OS cells expressing ectopic Hdm2 (Fig. 7A). In the absence of exogenous Hdm2, nucleolin stimulated p53 levels ~twofold (Fig. 7A, lanes 8 to 11, compare to lane 1). Expression of Hdm2 by itself reduced the amount of p53 (lane 2), and ectopic nucleolin expression in these cells increased the amount of p53 by ~4-fold over these lower levels (lanes 4 to 7; compare to lane 2). Unexpectedly, we also observed that nucleolin caused a marked reduction in the total level of Hdm2 (compare lane 7 to lane 2). This effect is studied in more detail below. Use of p53-null H1299 cells that were transiently transfected with p53 (Fig. 7B) again found that nucleolin caused a more significant p53 increase in cells also expressing ectopic Hdm2 (3.9-fold, compare lane 8 to lane 4). The greater effect of nucleolin on p53 levels in the presence of Hdm2 suggests that nucleolin inhibits Hdm2. If nucleolin did not act through Hdm2, nucleolin would be expected to cause a similar fold-increase of p53 in the presence or absence of Hdm2, even if the absolute levels of p53 differed.

- **Nucleolin inhibits Hdm2-mediated ubiquitination of p53 in vivo.** To test the hypothesis mentioned above, we characterized the effect of nucleolin on the Hdm2-mediated ubiquitination of p53 (Fig. 8A). In U2-OS cells, as expected, expression of ectopic Hdm2 resulted in the accumulation of poly-ubiquitinated p53 (lower panel, lane 2), which was further heightened by treatment with MG132 (lane 3). Strikingly, co-expression of nucleolin diminished p53 poly-ubiquitination, particularly at the higher nucleolin levels, and led to the formation of putative mono- and di-ubiquitinated p53 (lanes 4 to 5). Loss of the residual poly-ubiquitinated p53 at the higher levels of nucleolin correlated with an increase in the amount of p53. Interestingly, the inhibition of ubiquitination was persistent even in the presence of proteasomal inhibitor (lane 6). A similar loss of p53 poly-ubiquitination was observed in H1299 cells following high nucleolin expression, even after MG132 treatment (Fig. 8B, lower panel), and, to a lesser extent, in HCT116-wt cells that expressed both endogenous p53 and exogenous flag-tagged p53 (Fig. 8C, lower panel, compare lane 3 to lane 1). Control experiments found
that nucleolin did not appreciably alter the overall pattern of protein ubiquitination (data not shown). Hdm2 protein levels were diminished with ectopic expression of GFPNu compared to GFPc in U2-OS, H1299 and, to a lesser extent, HCT116wt cells (Fig. 8A to C). Our evidence indicates that nucleolin selectively disrupts p53 ubiquitination by Hdm2, resulting in p53 stabilization and an increase in cellular p53.

- **Nucleolin inhibits Hdm2-mediated ubiquitination of p53 in vitro.** To provide direct evidence that nucleolin affects p53 ubiquitination, we utilized an in vitro reconstitution system for p53 ubiquitination by Hdm2, using purified proteins (2). This system catalyzed robust p53 ubiquitination in an Hdm2-dependent reaction (Fig. 9; lane 2). While purified nucleolin had no effect on p53 modification in the absence of Hdm2 (lane 3), the addition of increasing amounts of nucleolin to Hdm2 dramatically reduced p53-ubiquitination (lanes 4 and 5). These results demonstrate that nucleolin inhibits Hdm2-mediated ubiquitination of p53 in vivo and in vitro.

- **Nucleolin is not a general inhibitor of E3 ubiquitin ligases.** p53 stability is governed by a balance between action of various E3 ubiquitin ligases including Hdm2 (3-7), COP1 (8), Pirh2 (9), and ARF-BP1/Mule (10), and ubiquitin-specific proteases that remove this post-translational modification (e.g., HAUSP (11, 12)). To provide additional evidence that nucleolin is not a general inhibitor of p53 ubiquitination, we tested HeLa cervical carcinoma cells. HeLa cells are transformed by human papillomavirus (HPV) type 18 and express the HPV E6 oncoprotein which, in conjunction with the cellular E6 associated protein (E6-AP; an E3 ubiquitin ligase), ubiquitinates p53 and destine it for proteolytic degradation (13). Over-expression of nucleolin in these cells had no significant effect on either p53 levels or the p53 ubiquitination state (Fig. 10A; lane 3), as compared to the empty vector control (lane 2). These data indicate that nucleolin does not inhibit p53 modification by E6/E6-AP. These data also lead to the conclusion that nucleolin does not have an associated ubiquitin protease activity similar to that of HAUSP (11, 12), as such activity would be expected to cause a loss of poly-ubiquitinated p53.

As a further examination of the specificity of nucleolin on p53 ubiquitination, we tested the effect on the degradation of p53 by p300. p300/CBP can stimulate p53 degradation (14), with this effect mediated by the E4 ubiquitin ligase activity of p300 (15). That is, mono-ubiquitin moieties on p53 are extended by p300 and yield poly-ubiquitinated p53. Transfection of

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Figure 9. Nucleolin inhibits ubiquitination of p53 by Hdm2 in vitro.

Figure 10. Nucleolin does not inhibit activities of other ubiquitin ligases.
SJSA cells with a plasmid expressing HA-p300 was found to cause the loss of p53 (Fig. 10B; lane 12 and data not shown). Co-transfection of either nucleolin (lanes 4 to 7) or empty vector (lanes 10 and 11) with p300 was unable to prevent this diminution in the level of endogenous p53. Nucleolin expression also had no effect on p300 levels (data not shown). These data indicate that over-expression of nucleolin does not inhibit p300 from stimulating p53 degradation. Similar results were observed in U2-OS as well as H1299 cells (data not shown). Although p300 has been reported to stimulate p53 poly-ubiquitination ([15]), we did not observe such effects in either the absence or presence of nucleolin (data not shown), using these cell lines and conditions.

• **Nucleolin associates with Hdm2 in vivo and in vitro.** The inhibition of p53 ubiquitination could be a consequence of nucleolin binding to the C-terminal regulatory region of p53 ([16]), which contains multiple lysines that are ubiquitination targets ([17]), and hence sterically block Hdm2 action. Alternatively, nucleolin might directly bind Hdm2 and thereby alter its ability to modify p53. As a first test of this hypothesis, we tested the interaction of endogenous nucleolin and Hdm2 in co-immunoprecipitation studies. In p53-positive U2-OS or p53-negative H1299 cells, use of either of two different antibodies to immunoprecipitate Hdm2 also co-precipitated nucleolin (Fig. 11A, lanes 2 and 3). The control IgG did not pull down either Hdm2 or nucleolin (lane 4). The reverse immunoprecipitation reaction involving anti-nucleolin antibodies also precipitated Hdm2 from extracts of these two cells lines as well as the SJSA line (which over-expresses endogenous Hdm2) (Fig. 11B). Similar results were found using ectopically-expressed nucleolin and Hdm2 (data not shown).

We also examined the ability of purified GST-Hdm2 to interact with nucleolin in lysates from HCT116-wt or -ko cells (i.e., wild-type or knockout for p53). Following incubation of GST-Hdm2 or GST alone with lysates, analysis of the complexed material found that nucleolin bound GST-Hdm2 (Fig. 12, lanes 3 and 4) but did not detectably associate with GST alone (lanes 1 and 2). We did not discern any influence of p53 on the ability of nucleolin to bind Hdm2 in this assay. In sum, these data indicate that nucleolin and Hdm2 can complex in vivo and in vitro in a p53-independent manner.

• **Nucleolin inhibits the Hdm2 ubiquitin ligase activity in vivo.** The ability of nucleolin and Hdm2 to physically interact suggests that nucleolin might inhibit the Hdm2 ubiquitination activity directly, thereby explaining the effect of nucleolin on p53 modification. To test this possibility, we examined Hdm2 auto-ubiquitination as an indicator of its overall ubiquitin ligase activity. In H1299 cells transfected with Hdm2 and His-tagged ubiquitin (Ub-His), a significant level of ubiquitinated Hdm2 was observed (Fig. 13A, lane 4). When these cells were co-transfected with increasing levels of nucleolin, the level of ubiquitinated-Hdm2 and total
Hdm2 (n.b., the ubiquitinated proteins were not isolated in this experiment) progressively declined (lanes 5 and 6). At the highest level of nucleolin, ubiquitinated-Hdm2 was barely detected (lane 6). Note that this level corresponds to only a ~twofold increase in total nucleolin (compare endogenous and GFP-tagged nucleolin in lane 6, lower panel). Because nucleolin has an effect on Hdm2 protein levels (described in greater detail below), we more rigorously tested the influence of nucleolin on Hdm2 auto-ubiquitination. H1299 cells were transfected with Hdm2, Ub-His, and two different levels of nucleolin (Fig. 13B). Aliquots that contained equivalent total Hdm2 protein were removed from each lysate, and then analyzed by SDS-PAGE and Western blotting to determine the level of modified Hdm2. Using this approach, we observed that heightened expression of nucleolin resulted in a loss of the ubiquitinated form of Hdm2 (compares lanes 2 and 3 to control lane 1). We conclude that nucleolin inhibits the auto-ubiquitination activity of Hdm2, and this reduced ubiquitin ligase activity contributes to decreased p53 ubiquitination.

- **Nucleolin diminishes Hdm2 protein levels.** As indicated above, nucleolin caused an apparent loss of Hdm2 protein, even though inhibiting Hdm2 auto-ubiquitination (a surprising observation because heightened auto-ubiquitination has been reported to destabilize Hdm2 (18)). We therefore further characterized the effect of nucleolin on Hdm2 levels in various cell lines. Because endogenous Hdm2 levels are normally very low, cells were transfected with Hdm2 and various amounts of the nucleolin expression constructs. In U2-OS cells, increases in the level of nucleolin caused a drastic reduction in Hdm2 protein levels (Fig. 14A, lanes 4 to 6), with Hdm2 found to be virtually undetectable at the highest level of nucleolin (lane 6). A similar decrease in Hdm2 protein levels was observed in H1299 cells, indicating that the presence of p53 was not required for nucleolin to mediate this effect (Fig. 14B; compare lanes 4 and 9). To elucidate if the nucleolin-Hdm2 interaction is influenced by the presence of p53, we directly tested the effect of p53 (Fig. 14C). Nucleolin again diminished the level of Hdm2 to an almost undetectable amount (Fig. 14C, compare lane 7 to lane 2). The down-regulation of Hdm2
expression by nucleolin was similar in the presence or absence of p53. Interestingly, upon nucleolin over-expression, total Hdm2 protein accumulation was slightly reduced in the presence of MG132 (Fig 14A, compare lane 7 to lane 3; Fig. 14C, compare lane 8 to lane 3). Overall, these data indicate that nucleolin reduces Hdm2 protein, with this reduction not strongly influenced by p53.

- **Nucleolin stimulates p53 transcriptional activity.** Because changes in the level of nucleolin can alter the amount of p53 protein, we determined the effect of nucleolin on various p53-mediated activities, first testing p53 transcriptional activity in H1299 cells. Using p53-responsive elements from the promoters of the cyclin-dependent kinase (cdk) inhibitor p21^{cip1/waf1} or Mdm2 genes, we found that nucleolin stimulated expression from 1.9 to 3.4-fold in the presence of p53 (Fig. 15A). Nucleolin in the absence of p53 had no significant effect. We also measured the expression of endogenous p21^{cip1/waf1} protein, and found that nucleolin increased the level of this key cell-cycle regulator (Fig. 15B). At the highest level of nucleolin, genotoxic stress did not markedly stimulate the expression of p53 or p21^{cip1/waf1} protein (Fig. 15B, compare lanes 4 and 5).

Further, when the isogenic cell lines HCT116-wt and -ko were examined, higher levels of p21^{cip1/waf1} were again seen following expression of GFP-nucleolin (Fig. 15C, lane 2). Only very low levels of p21^{cip1/waf1} were observed in HCT116-ko cells expressing GFP-nucleolin (Fig. 15C, lane 4), demonstrating that the stimulation is p53-specific. Similar results were noted for the pro-apoptotic Bax gene product (data not shown).

- **Nucleolin stimulates the activation of p53 in response to low levels of stress.** Testing the effect of nucleolin on the genotoxic stress response, we found that nucleolin expression enhanced p53 activation at low levels of CPT as compared to the corresponding vector control (Fig. 16, compare lanes 2 to 3 with lanes 6 to 7, respectively). A corresponding increase in p21 levels was similarly noted in cells expressing GFP-nucleolin compared to GFP alone. As CPT levels increased further, no significant differences in p53 and p21 levels was noted between these cells (lanes 4 and 8). As found above, Hdm2 levels were significantly reduced with nucleolin over-expression as

![Figure 15. Nucleolin stimulates p53-mediated transcription.](image1)

![Figure 16. Nucleolin stimulates p53 activation.](image2)
compared the vector-transfected cells. These data indicate that up-regulation of p53 levels by nucleolin can also lead to heightened expression of various p53-responsive genes.

- **Nucleolin inhibits cellular proliferation.** Because nucleolin increases p21cip1/waf1 expression, we examined the effect of nucleolin expression on cell proliferation. As a simple indicator of proliferation, we expressed either GFP or GFP-nucleolin in U2-OS or SJSA cells, plated the cells at equal low densities, and then grew the cells under G418 selection (Fig. 17A). Visual inspection found that cells expressing GFP-nucleolin had a clear growth disadvantage over cells expressing GFP. To quantitate this effect, we expressed nucleolin in HCT116-wt cells and observed that nucleolin inhibited cell growth to levels ~60% of these same cells transfected with the empty vector (Fig. 17B). In contrast, nucleolin expression in HCT116-ko cells had lesser effect as compared to cells transfected with an empty vector control, with this residual effect potentially due to the ability of nucleolin to inhibit the replication factor RPA (1, 19, 20). These data indicate that heightened expression of nucleolin inhibits cell proliferation in a p53-dependent manner.

- **Nucleolin increases cellular apoptosis.** Because p53 regulates numerous genes involved in the cellular apoptotic program, we determined if p53-mediated apoptosis was modulated by nucleolin. Stable clones of U2-OS cells were generated that expressed either GFP-nucleolin or GFP alone. Interestingly, although cells were maintained under G418 selection, it was found that GFP-nucleolin expression was lost in ~50% of cells following two weeks of growth, compared to ~25% of cells losing GFP expression (data not shown). Following selection, the fraction of GFP-positive cells undergoing apoptosis was then examined using a TUNEL assay (Fig. 18). 1500 cells were examined for each clone. Cells expressing GFP-nucleolin were found to have a ~twofold higher level of apoptosis (4.5%) compared to cells expressing GFP (2.5%). Our data indicate that heightened nucleolin expression can both inhibit cellular proliferation and increase apoptosis under normal growth conditions.
The above data are in press:


**Abbreviated summary of above Aim 3 work:** Our data implicate nucleolin as a key regulatory factor in the p53-Hdm2 circuitry in breast cells and in other tissues. The consequences of elevated p53 levels induced by nucleolin are enhanced expression of p21cip1/waf1, a corresponding reduction of cellular proliferation rate, and an increased rate of apoptosis. The nucleolin gene contains a c-Myc binding site (E-box) in the first intron and nucleolin transcription is stimulated ~4-fold by c-Myc, suggesting that it is directly responsive to proliferative signals (21). Indeed, nucleolin protein expression is coupled to the cellular growth rate with proliferating cells having a >3-fold higher nucleolin protein levels compared to quiescent cells (e.g., see ref. 22). Combined, these data indicate that nucleolin increases p53 protein levels in response to hyper-proliferative signals, and thereby provide a check against uncontrolled cellular growth in breast cells. We propose that nucleolin functions in an ARF-independent pathway to regulate p53 and Hdm2 in response to hyper-proliferative signals.

- **Nucleolin inhibits the Hdm2 ubiquitin ligase activity in vitro.** We are currently examining the effect of nucleolin on the Hdm2 ubiquitination activity in vitro using purified proteins. Basically, Hdm2 is incubated with E1 and E2 ubiquitination factors, and His-tagged ubiquitin (His-Ub). The reaction products are mixed with Ni²⁺-NTA beads to bind the ubiquitinated products. The bound material is then eluted, subjected to SDS-PAGE, and analyzed by Western blotting using anti-Hdm2 antibodies. An example of the assay is provided in Figure 19A. In the absence of His-Ub, no Hdm2 signal was observed showing that non-ubiquitinated Hdm2 does not associate with the Ni²⁺-NTA beads (lane 1). In the presence of His-Ub and GST, Hdm2 was subject to significant modification (lane 2). A large signal was observed at the position of native Hdm2 which is likely mono-ubiquitinated protein (‘mUb-Hdm2’). Above this band, a smear is seen indicative of poly-ubiquitinated Hdm2 (‘pUb-Hdm2’). We also observe a quickly migrating species that is due to prematurely-terminated Hdm2 (‘mUb-ΔHdm2’) that is likely mono-ubiquitinated by full-length Hdm2. In the presence of a GST-nucleolin (lane 3), we observed a modest reduction in the amount of pUb-Hdm2 and significant loss of mUb-ΔHdm2. These changes were analyzed by quantitating the signals from lanes 2 and 3 and comparing the traces (Fig. 19B; the difference is indicated by a dark grey fill between the two lines). These data indicate that nucleolin inhibits the ubiquitin ligase activity of Hdm2. We are planning to test domains of the nucleolin molecule in the near future.

Figure 19. Nucleolin inhibits Hdm2 autoubiquitination. Dark grey fill indicates differences between +/- nucleolin.
Task 4.
- **Effect of nucleolin on the Hdm2-mediated nuclear exclusion of p53 and on MDM2 localization.** We prepared vectors that allow expression in human cells of Hdm2 and nucleolin tagged with either yellow fluorescent protein (YFP) or cyan fluorescent protein (CFP), respectively. Using these and other tagged-nucleolin and Hdm2 variants, we have been unable to find any definitive evidence that nucleolin alters the localization of either p53 or Mdm2. We have therefore completed work on this aim.

Task 5.
- **To characterize the effect of nucleolin on MDM2–ERα complex formation in vitro, and on the stimulation of ERα transactivation by MDM2 in vivo.** Because of additional experiments required to complete the Oncogene study mentioned above, we are only beginning these experiments now.

The ability of nucleolin to modulate the p53-Hdm2 circuitry suggests a potential therapeutic route to prevent breast cancer development. Recent work has shown that, at the earliest stages of tumorigenesis, early pre-cancer lesions demonstrate a DNA damage response such as p53 activation, ATM activation, and phosphorylation of histone H2AX (23, 24). Thus, manipulating the levels and/or activity of nucleolin may allow clinicians to slightly increase the p53 surveillance machinery, and thereby reduce breast tumorigenesis.

Note that the results described above are the most important scientific results. In terms of our progress on specific technical aspects of the proposal, we have completed:

*Task 1 (~100% complete).*

*Task 2 (~75%; a to c complete; d is in progress).*

*Task 3 (~75% complete with sub-tasks a and b finished. Sub-tasks c, d, and e are in progress.*

*Task 4 (~100% complete. No further work on this aim is contemplated.)*

*Task 5 (Just beginning)*
Future work to continue the study of nucleolin and Hdm2 interaction

1) We have identified the nucleolin central RBD domain has having the highest activity in p53 stabilization. Because we wish to develop a compound mimicking nucleolin activity for treatment of breast cancer, it will be essential to obtain high-resolution structural information of the nucleolin-Hdm2 complex (most likely a complex containing only those domains that are necessary for the interaction). With this information in hand, we will use computer-aided drug design to generate 1st generation reagents and test these for their ability to inhibit Hdm2 E3 ubiquitin ligase activity in cultured cells.

2) The mechanism by which nucleolin causes a loss of Hdm2 protein remains unknown. Possible explanations include nucleolin inhibiting Hdm2 translation, or nucleolin altering Hdm2 half-life. Preliminary in vitro study indicates that nucleolin alters the Hdm2 auto-ubiquitination pattern, which could suggest that nucleolin facilitates Hdm2 export to the cytoplasm where it becomes degraded. Deciphering the mechanism is an important issue. Further study should reveal an interesting mechanism by which nucleolin regulates Hdm2 protein levels.
KEY RESEARCH ACCOMPLISHMENTS

- Nucleolin inhibits Hdm2-mediated ubiquitination of p53 in vivo.
- Nucleolin inhibits Hdm2 auto-ubiquitination in vivo and in vitro.
- Nucleolin reduces Hdm2 protein levels in vivo.
- Heightened nucleolin expression results in significant stabilization (increased half-life) of the p53 protein.
- Knockdown of endogenous nucleolin by RNAi methodology has the opposite effect of nucleolin overexpression, namely, p53 levels are reduced.
- The previous two points indicate that alterations in the amount of nucleolin in vivo cause parallel changes in the level of p53.
- Up-regulation of p53 levels by nucleolin causes heightened expression of various p53-responsive genes.
- Over-expression of nucleolin inhibits cell proliferation in a p53-dependent manner.
- Higher nucleolin expression increases cellular apoptosis in a p53-dependent manner, in non-stressed cells.
- In cells subjected to low levels of genotoxic stress, nucleolin facilitates p53 activation. Combined with the above points, our data indicate that nucleolin increases the tumor surveillance properties of p53.
REPORTABLE OUTCOMES

A. Manuscripts, abstracts, presentations

Manuscripts

Much of the work described above will published in the near future in Oncogene:


Nucleolin constructs made with DOD funding were subsequently used in work examining the ability of nucleolin to cause a checkpoint response. Thus, DOD funding was used in the following studies involving nucleolin:


A recent review cited DOD funding:


In all four cases, DOD funding was acknowledged.
Abstracts and presentations

Over the three years, we gave presentations at the:

1. Indiana University School of Medicine (Seminar). 4/6/04
2. University of Milan, Italy (Seminar). 4/28/04
3. Vanderbilt University (Seminar). 6/10/04
4. Genomic Integrity Meeting in Galway, Ireland (Abstract/Poster). 6/22/04
5. 12th International p53 Workshop, Dunedin, New Zealand (Abstract/Poster). 11/6-10/04

Support from the Department of Defense was publicized in each case.

Note that nearly all of the funding for these trips was supplied by non-DOD sources, allowing the DOD support to be used directly for laboratory research.
B. Development of cell lines

We have developed stable cell lines that express GFP-nucleolin and control GFP alone using the parental HCT116++ cell line.

C. Employment or research opportunities applied for and/or received based on experience/training supported by this award.

I recruited Checo Rorie, a talented minority (African-American) scientist with a Ph.D. from UNC-Chapel Hill, to work on the project. After ~18 productive months in the lab, he was accepted into the highly competitive SPIRE (Seeding Postdoctoral Innovators in Research and Education), back in North Carolina. He was a 2nd author on the Oncogene study mentioned above. He will also have another publication to be submitted in the upcoming year.
CONCLUSIONS

The major accomplishment of our studies is the finding that nucleolin stabilizes p53 by inhibiting the p53-antagonist Hdm2. The increase in p53 protein by nucleolin leads to higher expression of p21cip1/waf1, a reduced rate of cellular proliferation, and an increase in apoptosis. Nucleolin also facilitated p53 activation in response to low levels of genotoxic stress. The properties of nucleolin are strikingly similar in many respects to the tumor suppressor ARF including: 1) up-regulation in response to proliferative signals, 2) stabilization of p53 by associating with Mdm2, 3) inhibition of the E3 ubiquitin ligase activity of Mdm2, and 4) reduction in Hdm2 protein levels. Importantly, while ARF and nucleolin can associate, our observed effects of nucleolin on Hdm2 activity and p53 protein levels are not dependent upon ARF because they can occur in cells that lack detectable p14ARF mRNA and protein expression. We hypothesize that nucleolin functions in such an ARF-independent pathway to regulate p53 and Hdm2 in response to hyper-proliferative signals. Our data suggest that nucleolin, like ARF, is an important tumor suppressor in humans.

Because Hdm2 normally modulates p53 protein levels in breast cells (as well as cells from other tissues), academic and biotechnology investigators have been developing agents that regulate Hdm2 activity to inhibit breast cancer growth (e.g., Nutlin). Our finding that nucleolin controls both the level and activity of Hdm2 is of outstanding importance because it suggests that agents that mimic nucleolin activity could be used to prevent and inhibit breast cancer growth.
REFERENCES
APPENDICES

Four publications are attached that acknowledge DOD funding:


Nucleolin inhibits Hdm2 by multiple pathways leading to p53 stabilization

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Nucleolin is a c-Myc-induced gene product with defined roles in ribosomal RNA processing and the inhibition of chromosomal DNA replication following stress. Here we find that changes in nucleolin protein levels in unstressed cells cause parallel changes in the amount of p53 protein. Alterations in p53 levels arise from nucleolin binding to the p53 antagonist Hdm2, resulting in the inhibition of both p53 ubiquitination and Hdm2 auto-ubiquitination. Nucleolin does not alter p53 ubiquitination by human papillomavirus E6, indicating that the effect is specific for Hdm2. Although the inhibition of ligase activity would be expected to stabilize Hdm2, we instead find that nucleolin also reduces Hdm2 protein levels, demonstrating that nucleolin inhibits Hdm2 using multiple mechanisms. Increases in nucleolin levels in unstressed cells led to higher expression of p21\(^{\text{cip1/waf1}}\), a reduced rate of cellular proliferation, and an increase in apoptosis. Thus, nucleolin has a number of properties in common with the tumor suppressor ARF. We propose that nucleolin, like ARF, responds to hyperproliferative signals by upregulation of p53 through Hdm2 inhibition.

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Keywords: ;

Introduction

The transcription factor p53 exerts a pivotal role in controlling cell cycle progression and apoptosis in response to various forms of genotoxic and cellular stress (Anderson and Appella, 2004). Although normally existing in a latent state, stress conditions cause p53 to become activated, allowing transcriptional modulation of a large body of genes. Mice engineered with heightened p53 levels during development cause parallel changes in the amount of p53 protein. Alterations in p53 levels arise from nucleolin binding to the p53 antagonist Hdm2, resulting in the inhibition of both p53 ubiquitination and Hdm2 auto-ubiquitination. Nucleolin does not alter p53 ubiquitination by human papillomavirus E6, indicating that the effect is specific for Hdm2. Although the inhibition of ligase activity would be expected to stabilize Hdm2, we instead find that nucleolin also reduces Hdm2 protein levels, demonstrating that nucleolin inhibits Hdm2 using multiple mechanisms. Increases in nucleolin levels in unstressed cells led to higher expression of p21\(^{\text{cip1/waf1}}\), a reduced rate of cellular proliferation, and an increase in apoptosis. Thus, nucleolin has a number of properties in common with the tumor suppressor ARF. We propose that nucleolin, like ARF, responds to hyperproliferative signals by upregulation of p53 through Hdm2 inhibition.

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Introduction

The transcription factor p53 exerts a pivotal role in controlling cell cycle progression and apoptosis in response to various forms of genotoxic and cellular stress (Anderson and Appella, 2004). Although normally existing in a latent state, stress conditions cause p53 to become activated, allowing transcriptional modulation of a large body of genes. Mice engineered to lack p53 expression have a high propensity for development of a broad spectrum of tumors (Donehower et al., 1992). In humans, altered p53 regulation is a common step along the pathway of tumorigenesis with ~50% of human cancers showing mutation of the TP53 gene, often a loss of one gene copy and a point mutation within the second. p53 overexpression is also deleterious with heightened p53 levels during development causing organ atrophy (Nakamura et al., 1995; Godley et al., 1996; Allemand et al., 1999). One hypermorphic p53 mutant, although decreasing the susceptibility to tumor development, results in reduced longevity (Tyner et al., 2002). Control of the intracellular level of p53 therefore represents a critical feature for maintenance of normal cell proliferation and life span.

p53 activity is regulated by various means, including protein turnover. Stability of p53 is primarily mediated by the mouse double minute 2 (Mdm2) gene product (Brooks and Gu, 2004). Mdm2 (also known as Hdm2 in humans), an E3 ubiquitin ligase, directly interacts with and ubiquitinates p53, promoting its cytoplasmic degradation through the 26S proteasome (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997; Freedman and Levine, 1998; Roth et al., 1998). Mdm2 also regulates p53 by binding and occluding its N-terminal transactivation domain (Momand et al., 1992; Oliner et al., 1993). Evidence is emerging that p53 is also targeted by other ubiquitin ligases, including COP1 (Dornan et al., 2004), Pirh2 (Leng et al., 2003), ARF-BP1/Mule (Chen et al., 2005) and p300 (Grossman et al., 2003), and by ubiquitin-specific proteases such as HAUSP (Li et al., 2002, 2004), although the regulation of these enzymes is less clear.

Exposure to various stress stimuli cause alterations in the p53 modification state that facilitate loss of association with Mdm2, leading to higher p53 levels. In response to genotoxic stress, for example, p53 N-terminal sites are targeted by members of the phosphatidylinositol 3-kinase-like kinase (PIKK) family, including ATM, ATR and DNA-PK, as well as downstream effector kinases Chk1 and Chk2 (Anderson and Appella, 2004; Bode and Dong, 2004). Such modifications both reduce p53 proteolytic degradation and increase the accessibility of the transactivation domain, among other effects. Activation of these kinases also leads to heightened Mdm2 auto-ubiquitination and Mdm2 destabilization (Stommel and Wahl, 2004). The importance of Mdm2 in appropriately regulating p53 is demonstrated both by the lethality of Mdm2-null mice and the fact that viability can be rescued by simultaneous deletion of the p53 gene (Jones et al., 1995;
Montes de Oca Luna et al., 1995). In addition, a hypomorphic allele of Mdm2 exhibits increased p53 transcriptional activity and apoptosis in homeostatic tissue (Mendrysa et al., 2003). In humans, overexpression of Hdm2 is common in a variety of different tumor types, particularly in soft tissue tumors and osteosarcomas, that express wild-type (wt) p53 (Momand et al., 1998). These data attest to the conclusion that Mdm2 levels, like those of p53, must be exquisitely controlled.

A number of factors that alter the p53–Mdm2 circuitry have been identified. One prominent member of this group is the ARF tumor suppressor (p14ARF in humans, p19ARF in mice) (Lowe and Sherr, 2003). Oncogene overexpression increases ARF levels (de Stanchina et al., 1998; Palmero et al., 1998; Radfar et al., 1998; Zindy et al., 1998) and stimulates ARF–Mdm2 complex formation (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). This in turn causes a decrease both in Mdm2 auto-ubiquitination (indicative of reduced ubiquitin ligase activity) and in the ubiquitination of p53, thus stabilizing p53. Nevertheless, the increase in p53 levels in response to oncogene expression is only partially abrogated in ARF-null cells (de Stanchina et al., 1998; Zindy et al., 1998) and, in certain tumorigenesis models, p53 upregulation following oncogenic stress does not involve ARF (Tolbert et al., 2002; Verschure et al., 2002). Thus, ARF-independent pathways that stimulate p53 in response to hyperproliferative signals surely exist. A number of Mdm2-interacting proteins that regulate the degradation of p53 have recently come to light, including the tumor susceptibility gene 101 (TSG101 (Li et al., 1998)), the transcription factor Yin Yang 1 (YY1 (Gronroos et al., 2004; Sui et al., 2004)), and nucleophosmin (also called B23 (Kurki et al., 2004)), the latter protein also able to bind and inhibit ARF (Itahana et al., 2003; Bertwistle et al., 2004; Korgaonkar et al., 2005). A role for the nucleolus in the regulation of p53 has recently become apparent with nucleolar disruption leading to p53 stabilization (Rubbi and Milner, 2003; Olson, 2004). ARF has been found to sequester Mdm2 in the nucleolus (Weber et al., 1999), although this activity does not appear to be requisite for ARF-dependent p53 stabilization (Llanos et al., 2001; Korgaonkar et al., 2002).

Another prominent nucleolar phosphoprotein is nucleolin (also called C23 (Ginisty et al., 1999; Srivastava and Pollard, 1999)), an abundant c-Myc-induced gene product (Greasley et al., 2000). In response to c-Myc or a heightened cellular proliferation rate, nucleolin levels can increase ~4-fold (Sirri et al., 1997; Greasley et al., 2000). Nucleolin functions at an early step in precursor ribosomal RNA (rRNA) processing (Ginisty et al., 1998; Borovjagin and Gerbi, 2004), and disruption of nucleolin homologues in yeast cause unbalanced production of the large and small ribosome subunits (Kondo and Inouye, 1992; Lee et al., 1992; Gulli et al., 1995). Although the yeast strains lacking expression of the homologs are viable, they show severe defects in growth, strongly suggesting that knockout (ko) of the mammalian protein will be lethal. Nucleolin has stress-regulated interactions with numerous mRNA molecules (Yang et al., 2002), and regulates the turnover of particular mRNAs, including those encoding the apoptosis inhibitor Bcl-2 and the cell-cycle regulatory factor gadd45x (Sengupta et al., 2004; Zheng et al., 2005). Recently, nucleolin has been found to inhibit translation of the p53 message following DNA damage (Takagi et al., 2005). Nucleolin is also directly involved in the transcriptional regulation of various genes, including rRNA (Bouche et al., 1984; Egyhazi et al., 1988). In response to heat shock or genotoxic stress, nucleolin serves to inhibit chromosomal DNA replication by binding and repressing replication protein A (RPA) (Daniely and Borowiec, 2000; Wang et al., 2001; Kim et al., 2005), the cellular single-stranded DNA-binding protein. These stresses also cause a fraction of the nucleolin pool to relocalize from the nucleolus to the nucleoplasm in a reaction stimulated by physical interaction with p53, but independent of the ability of p53 to activate transcription (Daniely et al., 2002). Together, these data indicate that nucleolin globally modulates DNA and RNA metabolism following stress.

Here we examine the effect of nucleolin on the p53–Hdm2 regulatory loop. We find that nucleolin stabilizes p53 by binding and inhibiting Hdm2. Although the effects of nucleolin mimic those seen previously for ARF, nucleolin can exert these effects in cells lacking ARF expression. Because nucleolin levels are increased by proliferative signals, we propose that nucleolin acts in a pathway parallel to ARF to increase p53 levels in response to oncogenic stress.

**Results**

**Nucleolin modulates p53 levels in vivo**

To understand the significance of the previously identified nucleolin–p53 interaction (Daniely et al., 2002), we characterized the effect of nucleolin on p53 protein levels in cells that normally express wt p53 (U2-OS, HCT116-wt) or in p53-null cell lines (H1299 or HCT116-ko) transfected with wt p53. In contrast to H1299 cells, both U2-OS and HCT116 cells lack detectable expression of ARF (Park et al., 2002), and these expression patterns are not altered by nucleolin (data not shown). In the current study, we employ tagged-nucleolin constructs which have properties that are identical to those of endogenous nucleolin, including intracellular localization and ability to associate with RPA following genotoxic stress (Kim et al., 2005). Transfection of U2-OS cells with increasing levels of a plasmid expressing green fluorescent protein (GFP)-tagged nucleolin (GFPNu) was found to raise p53 levels nearly fourfold relative to mock-transfected cells and nearly to the level of p53 found in cells treated with
proteasome inhibitor MG132 (Figure 1a). There was an 
~2-fold increase in total nucleolin protein at the highest 
levels of nucleolin-vector transfected (data not shown; 
see Figure 7a), indicating that the effects on p53 levels 
are not a result of extreme nucleolin overexpression. 
Transient or stable expression of nucleolin increased the 
level of p53 in other cell lines, including HCT116-wt 
cells (Figure 1b). Expression of nucleolin tagged with e-
Myc or Flag epitopes instead of GFP resulted in similar 
increases in p53 levels (Figure S1 and data not shown). 
Our data demonstrate that nominal increases in 
nucleolin expression significantly elevate p53 levels in 
ungstressed cells.

Takagi et al. (2005) have observed that nucleolin can 
inhibit p53 translation by binding to an element in the 5' 
untranslated region of the p53 message. We do not 
observe repressive effects of nucleolin on p53 protein 
levels, except when high amounts of the nucleolin 
overexpression vector are transfected (data not shown). 
Under our conditions which employ moderate levels 
of nucleolin, the translation repression mechanism does 
not appear to play a major regulatory role.

It is possible that even a slight increase in nucleolin 
expression results in genotoxic stress, leading to p53 
activation and stabilization. A hallmark of human p53 
activation is phosphorylation of Ser15 by members of 
the PIKK family. Testing the status of Ser15, nucleolin 
increased the total p53 level by twofold, and the level of 
pSer15-p53 remained unaltered (Figure 1c, lanes 1 and 
3; see also darker exposure). A parallel increase in 
endogenous p21^\text{kip1/waf1} protein levels was also observed 
(additional p53-downstream targets are examined in 
more detail below). In contrast, when cells transfected 
with GFPc (empty vector) or GFPNu were treated with 
the DNA-damaging agent camptothecin (CPT), similar 
increases in both the total and pSer15-p53 were noted 
(lanes 2 and 4). These data indicate that nucleolin 
increases p53 levels in a pathway, at least in part, 
distinct from that utilized by cells undergoing genotoxic 
stress.

Downmodulation of nucleolin reduces p53 levels

To more clearly show that nucleolin causes coordinate 
changes in the level of p53, we employed two different 
nucleolin-specific short-interfering RNA (siRNA) mole-
cules, siNu1 and siNu2, to downmodulate nucleolin 
levels. At 24 h post-transfection, both siNu1 and siNu2 
molecules reduced the amount of nucleolin protein to 20 
and 40%, respectively, of that observed in cells treated 
with a control siRNA molecule directed against 
luciferase (siLuc) (Figure 2a; compare lanes 2 and 5 
with lane 1). Control experiments found that the level of 
nucleolin in untransfected cells was similar to that 
detected in siLuc-treated cells (compare lanes 1 and 8). 
Reductions in the amount of p53 comparable to that of 
nucleolin were observed when p53 was examined by 
Western blotting (Figure 2a). Similar effects were seen 
by immunofluorescence microscopy (Figure 2b). Using 
either siRNA molecule, cells with reduced nucleolin 
staining were also found to be deficient in p53. No overt 
changes in the localization of the residual p53 were 
noted and cell morphology remained apparently normal 
during the course of the experiment. At late times post-
transfection (> 72 h; data not shown), as the level of 
nucleolin returned to more normal levels, the relative 
increase of p53 was found to be slightly higher than that 
observed for nucleolin, p21^\text{kip1/waf1} protein levels 
were also increased and a significant amount of death was 
noted. As nucleolin downmodulation appears to be 
soomewhat toxic to cells, we postulate that this stress is 
responsible for the relative increase in p53 protein levels.

A reduction in translational repression of the p53 
message by nucleolin is also possible (Takagi et al., 
2005). Overall, we find that alterations in the level of 
nucleolin cause parallel changes in the amount of p53 
protein.

Figure 1 Nucleolin overexpression increases p53 protein levels. 
Lysates were prepared from (a) U2-OS cells that were transiently 
transfected with increasing levels of a nucleolin-expression plasmid 
(GFPNu; 0.3 and 0.6 µg in a six-well plate), or from (b) HCT116-wt 
cells that were either transiently or stably transfected with the 
GFPNu or GFPc (empty vector) plasmids, as indicated. When 
required, cells were treated with 30 µM MG132 for 4 h to inhibit the 
proteasome. Lysates were then subjected to SDS-PAGE and 
Western blotting with antibodies against GFP (to reveal GFP-
nucleolin), p53 and β-actin, the latter as a loading control. These 
experiments show that nucleolin expression increases p53 protein 
levels. (c) U2-OS cells were transiently transfected with either GFPc 
or GFPNu expression vectors. As indicated, cells were treated with 
2 μM CPT for 90 min to cause DNA damage. The lysates were 
blotted for p53 phosphorylated on Ser15 (pS15-p53), total p53 
(p53), p21^\text{kip1/waf1}, GFP and 
β-actin. In this panel, note that the anti-
GFP antibody recognizes GFP in lanes 1 and 2, and GFP-nucleolin 
in lanes 3 and 4. To conserve space, the appropriate bands from the 
Western blot were spliced into a single panel. The relative amount 
of p53 was calculated after correction for the amount of β-actin.
Nucleolin regulates the half-life of p53

Because p53 levels are primarily governed through the regulation of p53 stability, we examined the influence of nucleolin on the p53 protein half-life. Following transfection of HCT116-wt cells with either GFPNu or GFPc expression vectors, cells were treated with cycloheximide and then harvested at various times post-treatment. Probing the lysates for p53 protein levels indicated a longer p53 half-life in cells expressing GFP-nucleolin (Figure 3a). Densitometric analysis of the p53 levels, corrected for the level of β-actin in the same sample, confirmed this result (Figure 3b). In contrast, knockdown of nucleolin did not show any obvious destabilization of p53, compared to use of control siRNA molecules (data not shown), perhaps suggesting that nucleolin might use additional mechanisms to control p53 levels (see above). In sum, our data indicate that heightened nucleolin expression results in significant stabilization of the p53 protein.

Nucleolin inhibits Hdm2-mediated ubiquitination of p53 in vivo and in vitro

The primary pathway of p53 turnover involves ubiquitination by Hdm2 and subsequent proteasomal degradation. Because our data suggest that nucleolin might interfere with this pathway, we examined the effect of Hdm2 on the ability of nucleolin to modulate p53 levels. We observed that nucleolin had a more pronounced effect on p53 levels in U2-OS cells expressing ectopic Hdm2 (Figure 4a). In the absence of exogenous Hdm2, nucleolin stimulated p53 levels ~2-fold (Figure 4a, lanes 8–11, compare to lane 1). Expression of Hdm2 by itself reduced the amount of p53 (lane 2), and ectopic nucleolin expression in these cells increased the amount of p53 by ~4-fold over these lower levels (lanes 4–7; compare to lane 2). Unexpectedly, we also observed that nucleolin caused a marked reduction in the total level of Hdm2 (compare lane 7 to lane 2). This effect is studied in more detail below. Use of p53-null H1299 cells that were transiently transfected with p53 (Figure 4b) again found that nucleolin caused a more significant p53 increase in cells also expressing ectopic Hdm2 (3.9-fold, compare lane 8 to lane 4). The greater effect of nucleolin on p53 levels in the presence of Hdm2 suggests that nucleolin inhibits Hdm2. If nucleolin did not act through Hdm2, nucleolin would be expected to cause a similar fold increase of p53 in the presence or absence of Hdm2, even if the absolute levels of p53 differed.
Nucleolin associates with Hdm2 and inhibits the Hdm2 ubiquitin ligase activity

The inhibition of p53 ubiquitination could be a consequence of nucleolin binding to the C-terminal regulatory region of p53 (Daniely et al., 2002), which contains multiple lysines that are ubiquitination targets (Rodriguez et al., 2000), and hence sterically block Hdm2 action. Alternatively, nucleolin might directly bind Hdm2 and thereby alter its ability to modify p53.

As a first test of this hypothesis, we tested the interaction of endogenous nucleolin and Hdm2 in co-immunopre-
In precipitation studies, in p53-positive U2-OS or p53-negative H1299 cells, use of either of two different antibodies to immunoprecipitate Hdm2 also co-precipitated nucleolin (Figure 6a, lanes 2 and 3). The control IgG did not pull down either Hdm2 or nucleolin (lane 4). The reverse immunoprecipitation reaction involving anti-nucleolin antibodies also precipitated Hdm2 from extracts of these two cell lines as well as the SJSA line (which overexpresses endogenous Hdm2) (Figure 6b). Further, endogenous Hdm2 from either U2-OS or H1299 cells formed a complex with ectopic GFP-nucleolin (Figure 6c, lanes 3 and 6), but not with GFP alone (Figure 6c, lanes 2 and 5). Similarly, U2-OS and H1299 cells were transfected with both Flag-Hdm2 and GFP-nucleolin, or the corresponding empty vectors. Use of anti-Flag antibodies co-precipitated Flag-Hdm2 and GFP-nucleolin (Figure 6d, lanes 3 and 6). We consistently observe a slightly higher level of nucleolin–Hdm2 complex in H1299 cells as compared to U2-OS cells, even though the latter cell line has more endogenous Hdm2 protein (Figure S2). As mentioned, inhibition of p53-ubiquitination was also found to be more striking in H1299 cells than in U2-OS cells (Figure 5a and b, above). While these differences may merely be a consequence of the use of two different cell types, we note that H1299 cells are p53-negative/ARF-positive,
while U2-OS cells are p53-positive/ARF-negative. Finally, we examined the ability of purified glutathione-S-transferase (GST)–Hdm2 to interact with nucleolin in lysates from HCT116-wt or -ko cells. Following incubation of GST–Hdm2 or GST alone with lysates, analysis of the complexed material found that nucleolin bound GST–Hdm2 (Figure 6e, lanes 3 and 4) but did not detectably associate with GST alone (lanes 1 and 2). We did not discern any influence of p53 on the ability of nucleolin to bind Hdm2 in this assay. We are currently examining the interactions between p53, Hdm2 and nucleolin using purified components to more directly test the possibility that p53 inhibits nucleolin–Hdm2 association. In sum, these data indicate that nucleolin and Hdm2 can complex in vivo and in vitro in a p53-independent manner.

The ability of nucleolin and Hdm2 to physically interact suggests that nucleolin might inhibit the Hdm2 ubiquitination activity directly, thereby explaining the effect of nucleolin on p53 modification. To test this possibility, we examined Hdm2 auto-ubiquitination as an indicator of its overall ubiquitin ligase activity. In H1299 cells transfected with Hdm2 and His-tagged ubiquitin (Ub-His), a significant level of ubiquitinated Hdm2 was observed (Figure 7a, lane 4). When these cells were cotransfected with increasing levels of nucleolin, the level of ubiquitinated Hdm2 and total Hdm2 (n.b., the ubiquitinated proteins were not isolated in this experiment) progressively declined (lanes 5 and 6). At the highest level of nucleolin, ubiquitinated Hdm2 was barely detected (lane 6). Note that this level corresponds to only an ~2-fold increase in total

Figure 6  Nucleolin and Hdm2 interact in vivo. Lysates from non-transfected p53-wt (U2-OS, SJSA) and p53-null (H1299) cells, as indicated, were subjected to immunoprecipitation using (a) two different antibodies against Hdm2 (Ab-1 and SMP14), control IgG or (b) anti-nucleolin antibodies. The immunoprecipitates were subjected to Western analysis using anti-nucleolin, Hdm2, or β-actin antibodies. These data indicate that endogenous nucleolin and Hdm2 associate in a p53-independent manner. (c) U2-OS or H1299 cells were mock-transfected (Cont), or transfected with the empty GFPc (vec) or GFP-nucleolin (nucleolin) vectors, as indicated. (d) Similarly, U2-OS or H1299 cells were mock-transfected (no DNA control; ‘-‘), or transfected with the empty Flag and GFP vectors (vec), or GFP-Nu (nuc) and Flag-Hdm2 (hdm2) vectors. Aliquots of the resulting lysates were subjected to immunoprecipitation with anti-Hdm2 (panel C), or anti-Flag (panel D) antibodies. The immunoprecipitates, or aliquots of the original lysates, were immunoblotted with antibodies directed against nucleolin, Hdm2, the GFP or Flag tags, and β-actin. ‘ns’ indicates a non-specific band. These data indicate that endogenous and exogenous nucleolin and Hdm2 associate in a p53-independent manner. We note that control experiments using non-specific IgG were unable to precipitate either tagged or endogenous nucleolin or Hdm2. (e) Hdm2 and nucleolin interact in vitro. Whole-cell extracts from HCT116-wt and -ko cells were incubated with beads containing GST-alone or GST–Hdm2. Following binding and extensive washing, the bead-bound material was analysed by SDS–PAGE and Western blotting for nucleolin and GST. To conserve space, the appropriate bands from GST-alone (lanes 1 and 2) and GST–Hdm2 (lanes 3 and 4) were spliced into a single panel.
nucleolin (compare endogenous and GFPNu in lane 6, lower panel). Because nucleolin has an effect on Hdm2 protein levels (described in greater detail below), we more rigorously tested the influence of nucleolin on Hdm2 auto-ubiquitination. H1299 cells were transfected with Hdm2, Ub-His and two different levels of nucleolin (Figure 7b). Aliquots that contained equivalent total Hdm2 protein were removed from each lysate, and then analysed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and Western blotting to determine the level of modified Hdm2. Using this approach, we observed that heightened expression of nucleolin resulted in a loss of the ubiquitinated form of Hdm2 (compares lanes 2 and 3 to control lane 1). We conclude that nucleolin inhibits the auto-ubiquitination activity of Hdm2, and this reduced ubiquitin ligase activity contributes to decreased p53 ubiquitination.

**Nucleolin diminishes Hdm2 protein levels**

As indicated above, nucleolin caused an apparent loss of Hdm2 protein, even though inhibiting Hdm2 auto-ubiquitination (a surprising observation because heightened auto-ubiquitination has been reported to destabilize Hdm2 (Stommel and Wahl, 2004)). We therefore further characterized the effect of nucleolin on Hdm2 levels in various cell lines. Because endogenous Hdm2 levels are normally very low, cells were transfected with Hdm2 and various amounts of the nucleolin expression constructs. In U2-OS cells, increases in the level of nucleolin caused a drastic reduction in Hdm2 protein levels (Figure 7c).
levels (Figure 7c, lanes 4–6), with Hdm2 found to be virtually undetectable at the highest level of nucleolin (lane 6). A similar decrease in Hdm2 protein levels was observed in H1299 cells, indicating that the presence of p53 was not required for nucleolin to mediate this effect (Figure 7d, compare lanes 4 and 9). To elucidate if the nucleolin–Hdm2 interaction is influenced by the presence of p53, we directly tested the effect of p53 (Figure 7e). Nucleolin again diminished the level of Hdm2 to an almost undetectable amount (Figure 7e, compare lane 7 to lane 2). The downregulation of Hdm2 expression by nucleolin was similar in the presence or absence of p53. Interestingly, upon nucleolin overexpression, total Hdm2 protein accumulation was slightly reduced in the presence of MG132 (Figure 7c, compare lane 7 to lane 3; Figure 7e, compare lane 8 to lane 3). Experiments using cells in which nucleolin levels were downmodulated by RNA interference (RNAi) did not reveal any significant differences in Hdm2 stability compared to cells treated with a control siRNA (data not shown), perhaps indicating that nucleolin uses a mechanism distinct from control of Hdm2 stability to reduce Hdm2 levels. Overall, these data indicate that nucleolin reduces Hdm2 protein, with this reduction not strongly influenced by p53.

**Figure 8** Nucleolin stimulates p53-dependent p21cip1/waf1 expression. (a) H1299 cells were transfected with GFPNu (nucleolin) and/or or 3×Flag p53 plasmids, as indicated, and the Hdm2− or p21−promoter reporter constructs driving expression of firefly luciferase. The fold-change in firefly luciferase activity is plotted relative to the level of Renilla luciferase in the same extracts. All transfections were performed in triplicates in two independent experiments. The data indicated that p53 stimulated transcription from the Mdm2 promoter with a P<0.05 confidence criteria (**) using the paired Student’s t-test. (b) Lysates of U2-OS cells transfected with increasing levels of GFPNu (100, 300 and 900 ng) were analysed for GFP (i.e., nucleolin), p53, p21cip1/waf1 and β-actin by Western blotting. To generate a genotoxic stress response, cells were treated with 2μM CPT for 90 min (lane 5). The data indicate that overexpression of nucleolin in U2-OS cells increases the protein level of the p53 transcriptional target gene product, p21cip1/waf1. (c) Isogenic cell lines HCT116-wt and -ko were transfected with either GFP or GFPNu. Lysates were then subjected to Western blotting to reveal p53, p21cip1/waf1, GFP and β-actin. Note that the GFP panel is spliced with the antibody recognizing GFP in lane 1 and GFP-nucleolin in lanes 2, 3 and 4. (d) U2-OS cells transfected with either GFPNu or GFPpc were subjected to various concentrations of CPT (0.02, 0.2, 0.6μM) for 4h. The levels of p53, p21cip1/waf1, Hdm2 and β-actin in cellular lysates were then analysed by Western blotting. The data suggest that, at low levels of genotoxic stress, nucleolin enhances p53 activation, resulting in a parallel increase in p21cip1/waf1 protein levels.

**Nucleolin stimulates p53 transcriptional activity and inhibits cellular proliferation**

Because changes in the level of nucleolin can alter the amount of p53 protein, we determined the effect of nucleolin on various p53-mediated activities, first testing p53 transcriptional activity in H1299 cells. Using p53-responsive elements from the promoters of the cyclin-dependent kinase (cdk) inhibitor p21cip1/waf1 or Mdm2 genes, we found that nucleolin stimulated expression from 1.9- to 3.4-fold in the presence of p53 (Figure 8a). Nucleolin in the absence of p53 had no significant effect. We also measured the expression of endogenous p21cip1/waf1 protein, and found that nucleolin increased the level of this key cell-cycle regulator (Figure 8b). At the highest level of nucleolin, genotoxic stress did not markedly stimulate the expression of p53 or p21cip1/waf1 protein (Figure 8b, compare lanes 4 and 5). Further, when the isogenic cell lines HCT116-wt and -ko were examined, higher levels of p21cip1/waf1 were again seen following expression of GFP-nucleolin (Figure 8c, lane 8). A corresponding increase in p21 levels was similarly noted in cells expressing GFP-nucleolin compared to GFP alone. As CPT levels increased further, no significant differences in p53 and p21 levels was noted between these cells (lanes 4 and 8).
As found above, Hdm2 levels were significantly reduced with nucleolin overexpression as compared to the vector-transfected cells. These data indicate that upregulation of p53 levels by nucleolin can also lead to heightened expression of various p53-responsive genes.

Because nucleolin increases p21cip1/waf1 expression, we examined the effect of nucleolin expression on cell proliferation. As a simple indicator of proliferation, we expressed either GFP or GFP-nucleolin in U2-OS or SJSA cells, plated the cells at equal low densities, and then grew the cells under G418 selection (Figure 9a). Visual inspection found that cells expressing GFP-nucleolin had a clear growth disadvantage over cells expressing GFP. To quantitate this effect, we expressed nucleolin in HCT116-wt cells and observed that nucleolin inhibited cell growth to levels ~60% of these same cells transfected with the empty vector (Figure 9b). In contrast, nucleolin expression in HCT116-ko cells had lesser effect as compared to cells transfected with an empty vector control, with this residual effect potentially due to the ability of nucleolin to inhibit the replication factor RPA (Daniely and Borowiec, 2000; Wang et al., 2001; Kim et al., 2005). These data indicate that heightened expression of nucleolin inhibits cell proliferation in a p53-dependent manner.

Because p53 regulates numerous genes involved in the cellular apoptotic program, we determined if p53-mediated apoptosis was modulated by nucleolin. Stable clones of U2-OS cells were generated, which expressed either GFP-nucleolin or GFP alone. Interestingly, although cells were maintained under G418 selection, it was found that GFP-nucleolin expression was lost in ~50% of cells following 2 weeks of growth, compared to ~25% of cells losing GFP expression (data not shown). Following selection, the fraction of GFP-positive cells undergoing apoptosis was then examined using a TdT-mediated dUTP nick-end labeling (TUNEL) assay (Figure 9c). In all, 1500 cells were examined for each clone. Cells expressing GFP-nucleolin were found to have an ~2-fold higher level of apoptosis (4.5%) compared to cells expressing GFP (2.5%). Our data indicate that heightened nucleolin expression can both inhibit cellular proliferation and increase apoptosis under normal growth conditions.

Discussion

The ability to increase p53 protein levels through binding and inhibition of the p53-antagonist Hdm2 implicates nucleolin as a key regulatory factor in the p53–Hdm2 circuitry. The consequences of elevated p53 levels are enhanced expression of p21cip1/waf1, a corresponding reduction of cellular proliferation rate and an increased rate of apoptosis. Importantly, the nucleolin gene contains a c-Myc binding site (E-box) in the first intron and nucleolin transcription is stimulated ~4-fold by c-Myc, suggesting that it is directly responsive to proliferative signals (Greasley et al., 2000). Indeed, nucleolin protein expression is coupled to the cellular growth rate, with proliferating cells having a >3-fold higher nucleolin protein levels compared to quiescent cells (e.g., see Sirri et al., 1997). We observe significant effects on p53 levels when the amount of nucleolin is changed only ~0.5–2-fold. Combined, these data indicate that nucleolin increases p53 protein levels in response to hyperproliferative signals, and thereby provide a check against uncontrolled cellular growth.

The number of parallels between nucleolin and the ARF tumor suppressor are striking. First, similar to the nucleolin properties that we have described, ARF expression is upregulated in response to proliferative
signals (de Stanchina et al., 1998; Palmero et al., 1998; Radfar et al., 1998; Zindy et al., 1998). At these elevated levels, ARF stabilizes p53 by associating with Mdm2 (Kamijo et al., 1998; Pomerantz et al., 1998; Stott et al., 1998; Zhang et al., 1998). Second, ARF inhibits the E3 ubiquitin ligase activity of Mdm2 (Honda and Yasuda, 1999; Midgley et al., 2000), and has been reported to reduce Hdm2 levels (Zhang et al., 1998). Third, both factors function in rRNA processing, although nucleolin is instrumental in facilitating an early cleavage event (Ginisty et al., 1998), while p19ARF inhibits downstream processing steps, likely by interference with the nucleophosmin/B23 endoribonuclease (Savkur and Olson, 1998; Itahana et al., 2003; Sugimoto et al., 2003; Bertwistle et al., 2004). Fourth, as would be expected of proteins with ribosome biogenic functions, both nucleolin and ARF are primarily nucleolar (Ginisty et al., 1999; Zhang and Xiong, 1999; Weber et al., 2000).

Importantly, while ARF and nucleolin can associate (Weber et al., 2000), our observed effects of nucleolin on Hdm2 activity and p53 protein levels are not dependent upon ARF because they can occur in cells that lack detectable p14ARF mRNA and protein expression. It is worth emphasizing that ARF-null cells can still raise p53 upon ARF because they can occur in cells that lack detectable p14ARF mRNA and protein expression. It is worth emphasizing that ARF-null cells can still raise p53.

Because nucleolin has numerous activities in common with ARF, a reasonable question to ask is whether nucleolin is a tumor suppressor itself. While our data suggest that the answer to this question is likely to be yes, we are not aware of any cancer cells expressing nucleolin mutants. This may simply be a consequence of the critical role that nucleolin plays in rRNA maturation. Budding and fission yeasts deleted for the gene encoding a nucleolin homolog (NSR1 and gar2+, respectively) are viable, but show severe growth defects and have aberrant pre-rRNA processing (Kondo and Inouye, 1992; Lee et al., 1992; Gulli et al., 1995).

Similarly, our RNAi-mediated downmodulation of nucleolin causes toxic effects on cell viability (data not shown), likely resulting from an inability to properly process rRNA. These data indicate that mammalian cells lacking nucleolin will be non-viable, or at least have an extreme growth restriction. That said, it is conceivable that nucleolin mutants that have selective defects in the interactions with Mdm2 and p53 can be constructed and thus directly tested for their tumor suppressor properties.

We find that nucleolin binds Hdm2, inhibits its E3 ubiquitin ligase activity and reduces Hdm2 protein levels, and these collectively lead to an increase in p53 levels in non-stressed cells. Although we have previously observed that nucleolin can associate with the C-terminal domain of p53 (which is the target for ubiquitination (Rodriguez et al., 2000)), nucleolin–p53 complex formation is minimal unless cells are subjected to genotoxic stress (Daniely et al., 2002). Taken together, these data suggest that nucleolin regulates p53 by different pathways in unstimulated cells compared to cells undergoing genotoxic stress. Nucleolin–Hdm2 complex formation can occur under normal unstressed conditions, even though their predominant subcellular localization is different (nucleolar vs nucleoplasmic, respectively). A similar paradox was noted previously in our analysis of complexes between nucleolin and RPA (Kim et al., 2005), with the latter factor showing only weak localization to the nucleolus. Even so, use of fluorescence resonance energy transfer (FRET) demonstrated that RPA had significant association with particular nucleolin mutants in the nucleolus as well as the nucleoplasm. Because nucleolin is a highly mobile factor constantly shuttling from the nucleolus to nucleoplasm, association can also occur in the latter compartment. For Hdm2, like RPA, our immunoprecipitation studies suggest that the fraction of the nucleolin and Hdm2 pools interacting with each other is relatively small. Because we nevertheless find that nucleolin has effects on Hdm2 activity, this suggests that nucleolin–Hdm2 complex formation is predominantly transient in nature. The ability of nucleolin to both inhibit Hdm2 auto-ubiquitination and cause a reduction in Hdm2 protein levels is surprising. Heightened Mdm2 auto-ubiquitination has been found to stimulate its degradation (Stommel and Wahl, 2004); yet we find that nucleolin both inhibits auto-ubiquitination and decreases Hdm2 protein levels. Because the effect of nucleolin can be partly reversed by use of a proteasomal inhibitor (MG132; Figure 7c and e), it is possible that nucleolin facilitates Hdm2 degradation even though preliminary results have not revealed any obvious effects of nucleolin overexpression or knockdown on Hdm2 half-life (data not shown). Other mechanisms are possible. Although nucleolin is predominantly a nucleolar protein, it constantly shuttles between the nucleolus and cytoplasm (Borer et al., 1989). Thus, it is possible that nucleolin aids Hdm2 export to the cytoplasm and stimulates its degradation. Because nucleolin regulates the stability of specific mRNAs (e.g., Bcl-2 and gadd45α; Sengupta et al., 2004; Zheng et al., 2005), we do not rule out the possibility that nucleolin might also regulate translation of the Hdm2-encoding mRNA (as it regulates p53 translation following DNA damage in MCF7 cells; Takagi et al., 2005) or Hdm2 mRNA stability. The mechanism by which nucleolin inhibits the ubiquitin ligase activity of Hdm2 is similarly unclear. Complex formation with nucleolin may cause conformational changes in Hdm2 that inhibit the ubiquitin ligase reaction. Alternatively, the presence of nucleolin has the potential to sterically block the association of Hdm2 with the E2 ubiquitin-conjugating enzyme or p53. These and other possibilities are under investigation.

Previous data from our laboratory and others indicate that nucleolin can inhibit chromosomal DNA replication following heat shock and genotoxic stress via complex formation with the essential DNA replication factor RPA (Daniely and Borowiec, 2000; Wang et al., 2001; Kim et al., 2005). Thus, nucleolin can regulate cell
cycle progression both through a p53-independent pathway and the p53-dependent pathway described here. Recently, it has been shown that nucleolin inhibits translation of the p53 mRNA following DNA damage in MCF7 cells (Takagi et al., 2005). Combined with the defined involvement of nucleolin in rRNA processing (Ginisty et al., 1998; Saez-Vasquez et al., 2004), these findings show nucleolin to be a central factor that integrates cellular processes, including ribosome biogenesis, proliferation and the response to stress.

More generally, it is now becoming obvious that a number of proteins involved in ribosome biogenesis regulate Mdm2 activity or modulate the Mdm2-antagonist ARF. In addition to nucleolin, factors such as nucleophosmin/B23 (Ithana et al., 2003; Bertwistle et al., 2004; Kurki et al., 2004; Korgaonkar et al., 2005) and the large ribosomal subunits L5, L11 and L23 (Lohrum et al., 2003; Zhang et al., 2003; Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004) have each been found to inhibit Mdm2. It has been estimated that pre-rRNA synthesis accounts for ~40–60% of total cellular transcription (Hannan et al., 1998), while more than half of total protein synthesis is devoted towards ribosome production (Moss, 2004). Cellular transformation has been suggested to proceed in step with deregulated protein synthesis (see, e.g. Ruggero and Pandolfi, 2003) and references therein). It would not be unexpected that inordinately high demands for protein synthesis as found within incipient cancer cells result in any significant changes to either the cellular or nucleolar appearance, and did not cause any apparent toxic effects on cells. When required, CPT (Sigma-Aldrich; a stock concentration of 10 mM in DMSO) was directly added to the growth media to a final concentration of 2 μM for 90 min. To determine the p53 half-life, HCT116-wt cells were transfected with either GFPc or GFPNu vectors. At 36 h post-transfection, cells were treated with cycloheximide (200 μg/ml), and harvested at various times. Lysates were prepared and analysed by Western blotting with anti-p53 antibodies (DO-1).

To generate stable cell lines, U2-OS cells were seeded at 5 × 10⁴ cells per 60-mm plate and transfected with 1 μg of GFPNu or 0.5 μg of GFPc vectors. Post-transfection (18h), the cells were replated at the density of 10⁶ cells/10 cm dish in duplicates. GFP-expressing cells were selected in McCoy’s 5A media containing 400 or 800 μg/ml G418 (Cellgro) medium for 24 days, with the drug-containing media replaced every week. Individual clones were subsequently isolated and expanded over a 2–4-week period.

For nucleolin downmodulation experiments, siRNA duplexes (InvivoGene) corresponding to nucleolin positions 2215–2235 (siNu1) or 2292–2310 (siNu2; in the 3’ untranslated sequence) were employed, using nucleolin sequence information from Accession NM_005381. U2-OS cells in 24-well plates were transfected with 200 nM of either siNu1 or siNu2, and then harvested at 24, 48 and 72 h post-transfection. Luciferase siRNA (siLuc) was used as a control. Immunofluorescence microscopy was performed as described by Vassin et al. (2004).

Materials and methods

Nucleolin, p53 and Hdm2 expression vectors
The expression constructs for human nucleolin full length (aa 1–707) containing an N-terminal GFP- (GFPNu), Myc or Flag-tag were described previously (Kim et al., 2005). To generate suitable vectors for stable expression in the HCT116 cell lines, the puromycin-resistance gene was cloned into GFPNu and GFPc (GFP-only; pEGFP-C1 from BD Biosciences Clontech) expression vectors. GST–Hdm2 was induced and expressed from Escherichia coli BL21 cells using the pGEX6P (Amersham Pharmacia) vector. The Flag-p53 wt (human), Hdm2 wt and His6-tagged ubiquitin (Ub-His) constructs were kindly provided by M Oren (Weizmann Institute), B Vogelstein (John Hopkins University) and M Pagano (New York University School of Medicine, New York, NY, USA), respectively.

Antibodies

The primary antibodies used for immunoprecipitation or Western blotting were as follows: nucleolin, either the mouse monoclonal MS-3 or rabbit polyclonal H250 (Santa Cruz Biotechnology); GFP, the rabbit polyclonal anti-GFP antibody (Molecular Probes); p53, mouse monoclonal DO-1 (Santa Cruz Biotechnology); p53 phosphorylated on Ser15, rabbit polyclonal pS15p53 (Cell Signaling Technology); p21, mouse monoclonal CIP1 (BD Biosciences Pharmingen); and Hdm2, mouse monoclonal SMP14 (Santa Cruz Biotechnology) or Ab1 (Oncogene Res. Products). Mouse monoclonal antibodies against GST, FLAG (M2) and β-actin were obtained from Sigma-Aldrich. The secondary antibodies used were anti-mouse and anti-rabbit HRP-conjugated antibodies (Amersham), and fluorescein-conjugate antibodies (Jackson ImmunoResearch).

Cell culture and transfection
HCT116 p53-wt and -ko cell lines (Bunz et al., 1998) were kindly provided by Dr Bert Vogelstein (Johns Hopkins University). All other cell lines were obtained from ATCC. Plasmid transfections were performed using Effectene transfection reagent (Qiagen). Nucleolin overexpression did not result in any significant changes to either the cellular or nucleolar appearance, and did not cause any apparent toxic effects on cells. When required, CPT (Sigma-Aldrich; a stock concentration of 10 mM in DMSO) was directly added to the growth media to a final concentration of 2 μM for 90 min. To determine the p53 half-life, HCT116-wt cells were transfected with either GFPc or GFPNu vectors. At 36 h post-transfection, cells were treated with cycloheximide (200 μg/ml), and harvested at various times.

When required, cells were treated with the proteasome inhibitor MG132 (30 μM; Calbiochem) for 4 h prior to harvest. Following washing with 1 × phosphate-buffered saline (PBS), cells were lysed directly on 100 mm dishes in 50 mM Tris-HCl, pH 7.6, 0.5 mM ethylene diamine tetraacetate (EDTA), 1% (w/v) SDS and 1 mM dithiothreitol (DTT), scraped into ependorf
In vitro ubiquitination was performed as described by Wang et al. (2002). Reactions (15 µl) containing bacterially expressed human E1, E2 (GST-UbcH5), p53 (1 µl produced in a wheat germ transcription-coupled in vitro translation system (Promega)), GST-Hdm2 (400 ng) and 10 µg ubiquitin (Sigma), GST-nucleolin (Kim et al., 2005), 1 µg/ml ATP/S (Boehringer), 40 mM Tris-HCl, pH 7.5, 2 mM DTT, and 5 mM MgCl2 were incubated for 60 min at 30°C. Reactions were quenched by addition of 15 µl of SDS-PAGE buffer, boiled for 5 min and analysed by 8% SDS-PAGE and Western blotting with an anti-p53 antibody (DO-1).

Immunoprecipitation and GST-pulldown
Transfected cells were lysed in 20 mM N-2-hydroxethylpiperazine-N'-2-ethanesulfonic acid, pH 7.4, 100 mM NaCl, 0.5% (v/v) Nonidet P (NP)-40, 10% (v/v) glycerol, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 0.1 mM Na3VO4, 1 mM NaF, and 1 µg per ml each of aprotinin, leupeptin and pepstatin. Cell extracts were incubated with the desired primary antibody for 2 h at 4°C, and the immunocomplex captured using either protein A or protein G-plus beads at 4°C overnight. The beads were then washed five times with BC100 buffer (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 10% glycerol, 100 mM KCl, 4 mM MgCl2, 0.2 mM PMSF, 1 mM DTT and 0.25 µg per ml of pepstatin), eluted with 2 × SDS-PAGE lysis buffer and boiled for 10 min. The proteins were resolved by SDS-PAGE gels and analysed by Western blotting.

To evaluate Hdm2 and nucleolin binding in vivo, a GST pulldown assay was performed. GST–Hdm2 and GST-alone were purified to near homogeneity on glutathione-Sepharose beads. Whole-cell extracts were prepared from HCT116-wt and -ko cells in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% (v/v) NP-40, 1% (v/v) Triton X-100, 2 mM DTT, 2 mM PMSF and 1 µg per ml each of aprotinin, leupeptin and pepstatin). Prior to the binding assay, lysates were diluted 10-fold with lysis buffer containing 200 mM NaCl but lacking NP-40 and Triton X-100 (i.e., to reduce the NP-40 and Triton X-100 levels to 0.1% (v/v) each), and then pre-cleared thrice over the glutathione-Sepharose beads before being loaded onto the GST–Hdm2 or GST-alone affinity columns. An equivalent amount of GST–Hdm2- or GST-bound beads were loaded with equal amount of protein from HCT116-wt or -ko cell lysates and incubated at 4°C overnight. The beads were washed extensively with 25 mM Tris-HCl, pH 7.8, 500 mM NaCl, 1 mM EDTA, 1 mM DTT, 0.1% (v/v) glycerol, 0.2% (v/v) NP-40, 1% (v/v) Triton X-100 and 0.1% sodium deoxycholate, and then equilibrated with 25 mM Tris-HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1 mM DTT, 10% (v/v) glycerol and 0.2% (v/v) NP-40 for loading. Proteins bound to the beads were analysed by Western using anti-nucleolin and anti-GST antibodies.

Western blot analysis
To visualize total cellular proteins, the cell suspension was pelleted and lysed in NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, pH 8.0, 1% (v/v) NP-40, 2 mM DTT, 0.1% PMSF), and incubated on ice for 30 min. Lysates were similarly obtained from the Hdm2 ubiquitination experiments. Proteins were subjected to Western blot analysis using standard conditions and visualized using ECL-Plus (Perkin-Elmer). Digital images were analysed using Image SXM and ImageJ software. β-Actin was used for normalization when quantitating band intensities.

Transcription assays
Plasmids encoding firefly luciferase under the control of the p21 or Hdm2 promoter were kindly provided by M Oren. Briefly, p21 and Hdm2 promoter sequences (both of which contain a p53 response element) were amplified by PCR and ligated into pGL3-Basic reporter plasmid (Promega), upstream of the luciferase gene. H1299 cells were transfected with Flag-p53 and/or GFPNu plasmids, along with either the p21-Luc or Hdm2-Luc reporter constructs. Renilla luciferase under the control of cytomegalovirus (CMV) promoter was cotransfected as a control. Cells were harvested 24 h post-transfection and luciferase assayed with a commercial double luciferase kit (Promega), employing a TD-20e luminometer (Turner BioSystems). Luciferase activities were normalized against the level of Renilla luciferase in the same extracts.

Proliferation assay
For visual examination of proliferation rates, U2-OS and SJSA cells stably transfected with GFPNu or GFPc cells were plated at constant density and grown for 3–4 weeks under G418 selection. Cells were fixed with 10% T-cell antigen for 20 min at room temperature, and then stained with crystal violet (0.5 in 80% methanol) for 15 min. For assessing the effect of nucleolin expression on growth rate, HCT116-wt and -ko cell lines were transiently transfected with Flag-nucleolin or empty Flag vectors. At 24 h post-transfection, cells were counted and replated at equivalent densities into 96-well plates in triplicate. Cells were harvested at different time points (40, 48, 72, 96 and 120 h post-transfection) and stained with 0.1% crystal violet in 80% methanol. After the cells were washed thoroughly with 1 × PBS, the crystal violet was eluted in absolute ethanol and measured at 600 nm in a 96-plate reader (Dynatech MR7000) as a measure of cell density. The experiment was performed on three independent occasions in triplicate. The relative growth rates were determined at each time point and averaged.

TUNEL
Apoptosis was assayed by TUNEL using the In Situ Cell Death Detection Kit (TMR Red; Roche) following the manufacturer’s protocol. Following end-labeling, the cells were viewed by fluorescence microscopy to visualize the TUNEL stain, GFP and Hoechst. GFPc- or GFPnu-positive cells with either complete or granular nuclear TUNEL staining were considered as positive apoptotic cells. In total, 1500 cells were counted for each construct.

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Novel Checkpoint Response to Genotoxic Stress Mediated by Nucleolin-Replication Protein A Complex Formation

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Human replication protein A (RPA), the primary single-stranded DNA-binding protein, was previously found to be inhibited after heat shock by complex formation with nucleolin. Here we show that nucleolin-RPA complex formation is stimulated upon genotoxic stresses such as treatment with camptothecin or exposure to ionizing radiation. Complex formation in vitro and in vivo requires a 63-residue glycine-arginine-rich (GAR) domain located at the extreme C terminus of nucleolin, with this domain sufficient to inhibit DNA replication in vitro. Fluorescence resonance energy transfer studies demonstrate that the nucleolin-RPA interaction after stress occurs both in the nucleoplasm and in the nucleolus. Expression of the GAR domain or a nucleolin mutant (TM) with a constitutive interaction with RPA is sufficient to inhibit entry into S phase. Increasing cellular RPA levels by overexpression of the RPA2 subunit minimizes the inhibitory effects of nucleolin GAR or TM expression on chromosomal DNA replication. The arrest is independent of p53 activation by ATM or ATR and does not involve heightened expression of p21. Our data reveal a novel cellular mechanism that represses genomic replication in response to genotoxic stress by inhibition of an essential DNA replication factor.

Genomic stability requires that cell cycle progression is tightly regulated and can be blocked at key transitions in response to genotoxic stress (38). In response to such stresses, eukaryotic cells activate pathways that both prevent entry into S phase and inhibit DNA synthesis in cells currently undergoing replication. Whereas certain mechanisms have been identified that, for example, block kinases necessary for S-phase progression (e.g., references 10, 11, and 19), other inhibitory pathways likely exist. Study of replication protein A (RPA), the primary single-stranded DNA-binding protein in eukaryotes (31, 57), has shown that this factor is a target for inactivation in response both to genotoxic stress and heat shock (8, 13, 36, 37, 52, 54, 55). However, the mechanisms of inactivation remain poorly understood.

RPA is composed of three distinct subunits of ~70 (RPA1), 30 (RPA2), and 14 (RPA3) kDa and is an essential factor in many DNA processing reactions. Genetic and biochemical studies demonstrate that RPA has required roles both in the initiation and in the elongation stages of DNA replication (31, 57). Similarly, RPA is necessary for homologous recombination and for DNA repair events that use the recombination machinery (for example, see reference 53 and references therein). It is also indispensable for nucleotide excision repair (1). Along with stabilizing DNA in its single-stranded form, RPA supports the activity of other factors through obligate interactions. For example, simian virus 40 (SV40) DNA replication can be reconstituted with RPA of a metazoan origin but not with Saccharomyces cerevisiae RPA (6, 39). RPA is integrally involved in the cellular checkpoint response as RPA recruits the ATR-ATRIP complex to sites of DNA damage and supports activation of the ATR kinase (59). RPA also recruits the replication factor C-like Rad17 complex to various DNA structures and assists the binding of the Rad9-Rad1-Hus1 complex (60).

As would be expected of a protein with multiple roles in DNA metabolism and in the response to DNA damage, RPA activity is regulated at various levels. The RPA2 subunit of RPA becomes phosphorylated in response to genotoxic stress by phosphatidylinositol 3-kinase-related kinases, including ATM and DNA-PK (see citations within references 5 and 52). Mutational analysis of the RPA2 phosphorylation sites indicates that RPA phosphorylation prevents recruitment of RPA to replication centers while having no effect on localization to sites of DNA damage (52). Downregulation of RPA activity also occurs by apparent phosphorylation-independent mechanisms. The most clearly identified pathway involves the inhibition of RPA activity by association with the nucleolar factor nucleolin (13, 54).

Nucleolin is an abundant protein that is required for the first step of pre-rRNA processing (22). Mutation of the genes encoding nucleolin homologues in budding and fission yeast disrupts balanced production of the small and large ribosomal subunits (24, 34, 35). Nucleolin has many other diverse activities, including regulation of transcription (20, 23, 26, 45, 58), modulation of mRNA stability (9, 48), and acting as a low-affinity receptor for human immunodeficiency virus on the cell surface (7, 41). In response to DNA damage conditions or heat shock, a significant fraction of the nucleolin pool relocates from the nucleolus to the nucleoplasm in a process stimulated by physical association with p53 (13, 14, 54). After heat shock, nucleolin-RPA complex formation is greatly stimulated, and
formation of this complex is inhibitory to DNA replication in vitro (13, 54). In vivo, the mobilized nucleolin sequesters RPA at sites distinct from replication centers (13). The mobilization of nucleolin in response to heat shock thus represents a novel pathway for regulating DNA replication.

We examined the interaction of nucleolin and RPA in response to DNA damage. We found that, like heat shock, genotoxic stress strongly induces nucleolin-RPA complex formation. The RPA-interacting domain was localized to the 63-amino-acid (aa) glycine-arginine-rich (GAR) domain at the extreme C terminus of nucleolin. Expression of GAR or a nucleolin mutant with constitutive association with RPA causes a block in the cellular transit from G1 into S phase. The nucleolin-mediated inhibition of chromosomal DNA replication could be prevented by overexpression of RPA2 to increase the cellular level of RPA. These data demonstrate a novel intra-S-phase checkpoint response in response to genotoxic stress through target of RPA by mobilized nucleolin.

MATERIALS AND METHODS

Construction of nucleolin and RPA2 expression vectors. For in vitro studies, human nucleolin and mutant nucleolin derivatives were expressed in Saccharomyces cerevisiae with N-terminal glutathione S-transferase (GST) tags and were purified as described below. The pKG-derived yeast plasmids that express full-length nucleolin (FL; aa 1 to 707), the N-terminal half of nucleolin (NT; aa 1 to 323), and the C-terminal half of nucleolin (CT; aa 323 to 707) were kindly provided by E. Rubin (University of Medicine and Dentistry of New Jersey [UMDNJ]). Other nucleolin variants, including the combined N terminus and first RNA-binding domain (RBD) (NT/RBD; aa 1 to 390), the combined N terminus and the complete RBD region (NT/RBD1-4; aa 1 to 648), and the C-terminal GAR domain (GAR; aa 645 to 707), were inserted into the pKG vector by using standard PCR-mediated cloning procedures.

For in vivo studies, nucleolin or nucleolin derivatives were expressed with N-terminal green fluorescent protein (GFP), cyan fluorescent protein (CFP), or Myc epitope tags. GFP and CFP fusion proteins were constructed by using PCR cloning into the pEGFP-C1 or pECFP-C1 vectors (Clontech). Similarly, Myc-tagged nucleolin (FL or mutants) was expressed from the pEFP/Myc-HisA plasmid (Invitrogen), as modified by Vassin et al. (52) to prevent expression of the His tag or, for proliferation studies, from a modified pEGFP-C1 vector in which the GFP tag was replaced by the Myc tag. Human RPA2 containing an N-terminal yellow fluorescent protein (YFP) tag was generated by excising the GFP tag was replaced by the Myc tag. Human nucleolin and mutant nucleolin derivatives were expressed in S. cerevisiae JEL1 strain with the appropriate plasmid, cells were grown in synthetic defined (SD) medium under selection in 2% raffinose, and protein expression was induced by 2% galactose. Extracts from these cultures were made by disruption of the yeast cells by using 25- to 50-μm glass beads in uracil RIPA buffer (50 mM Tris- HCl [pH 7.2], 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% sodium deoxycholate) with protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 7.2 mM, 150 mM NaCl, 0.1% sodium dodecyl sulfate [SDS], 1% Triton X-100, 1% dithiothreitol [DTT]). Glutathione-Sepharose beads (Pharmacia) were then added to the clarified yeast extract, followed by incubation to bind the GST-nucleolin proteins. After three washes with a 10× bead volume of RIPA buffer, the GST-tagged proteins were eluted with 10 mM reduced glutathione and 50 mM Tris-HCl (pH 7.5). After overnight dialysis at 4°C against phosphate-buffered saline (PBS) and 20% glycerol, eluates were assayed for purity by SDS-polyacrylamide gel electrophoresis (PAGE) and Coomassie blue staining.

The human RPA heterotrimer was produced in Escherichia coli BL21 transformed with the p11drRPA vector and purified as described previously (29, 30).

Far-Western analysis. Far-Western blotting was carried out basically as described by Jayaraman et al. (32). Purified GST-tagged nucleolin (FL and mutants) proteins were subjected to SDS-PAGE and then transferred to a nitrocellulose membrane. After two incubations in denaturation buffer (6 M guanidine-HCl in PBS) for 5 min at 4°C, the membrane was incubated six times in serial dilutions (1:1 [vol/vol]) of denaturation buffer, each dilution being with PBS containing 1 mM DTT. The membrane was blocked with PBS containing 0.1% Tween 20 (PBS-T) and 5% nonfat dry milk (NFDM) for 45 min at room temperature and washed twice with PBS-T and 0.25% NFDM. The membrane was then incubated with purified human RPA (0.2 μg/ml) in PBS-T, 0.25% NFDM, 1 mM DTT, and 2.5 mM phenylmethylsulfonyl fluoride for 2 h at room temperature and subsequently washed four times in PBS-T and 0.25% NFDM. The presence of bound RPA was probed by using a mouse anti-RPA2 monoclonal antibody (SSB34A; NeoMarkers) and horseradish peroxidase-conjugated sheep anti-mouse antibody as the primary and secondary antibodies, respectively, and detected by using enhanced chemiluminescence (Amersham Biosciences).

In vitro DNA replication assay. The SV40-based in vitro DNA replication assay was described previously (52) and utilized a pBlueScript SK+ phagemid (Strатегени) containing a 90-bp SV40 origin region (positions 5186 to 52) subcloned into the BamHII and Xhol sites (pBSor). Reaction mixtures (25 μl) contained the following: 40 mM HEPEs (pH 7.5); 40 mM creatine phosphate; 7 mM MgCl2; 0.5 mM DTT; 4 mM ATP; 200 μM concentrations of each CTP, GTP, and UTP; 100 μM concentrations each of dATP, dGTP, and dTTP; 40 μM [α-32P]UTP (3,000 cpm/mmol); Perkin-Elmer Life Sciences); 1.25 μg of creatine phosphokinase; 150 ng of pBS-or; 100 μg of AS65 protein fraction prepared from HeLa cells; 200 ng of RPA; 200 to 400 ng of the GST fusion proteins; and 500 ng of SV40 large T antigen. The reaction mixtures were first preincubated on ice for 30 min without the addition of plasmid DNA, deoxyribonucleoside triphosphates, ATP, and creatine phosphokinase. After the addition of remaining factors, the complete reaction was incubated at 37°C for 2 h. The replication activity was determined by precipitating the high-molecular-weight DNA with trichloroacetic acid and quantitating the amount of incorporated radioactivity in the precipitate by liquid scintillation counting.

Immunoprecipitation and immunoblotting. Plated U2-OS cells were transfected with 1 μg of specified expression plasmids by using Effectene transfection reagent (Qiagen). The transfected cells were harvested at 24 h posttransfection when visualized at 24 h posttransfection. When required, cells were either treated with 1 μM CPT or 2.5 mM hydroxyurea or exposed to 10 Gy of ionizing radiation or 30 J of UV light m⁻². The immunoprecipitation reaction was carried out by using the IMMUNOCatcher kit (CytoSignal) according to the manufacturer's instructions. Immunoprecipitated proteins were separated by using SDS–10% PAGE and transferred to a nitrocellulose membrane (Schleicher & Schuell). After incubation with the appropriate primary antibody, the membrane incubated with a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit secondary antibody, and the presence of bound proteins was detected with ECLplus (Amersham Pharmacia Biotech). The following antibodies were used for both detection and immunoprecipitation: RPA2, mouse monoclonal antibody SSB34A (NeoMarkers); RPA1, mouse monoclonal antibody Ab-1; nucleolin, mouse monoclonal antibody MCA9 (Southern Biotechnology Associates); and the GAR domain (Santa Cruz Biotechnology); GFP, rabbit polyclonal antibody (Molecular Probes); Myc, rabbit polyclonal antibody (Upstate Biotechnology); p53, DO-1 monoclonal antibody (Santa Cruz Biotechnology); (pSer15)p53, rabbit polyclonal antibody (Cell Signaling Technology); and p21, mouse monoclonal C1/p/WAF1 antibody (BD Biosciences/Pharmigen).

Immunofluorescence microscopy. To prepare for imaging, U2-OS cells grown on fibronectin-coated coverslips (BD Biotechnology) were treated as described previously (15). Cells were fixed for 20 min at room temperature with 4% (wt/vol) formaldehyde in PBS, permeabilized with 0.5% Triton X-100, rinsed with PBS, and then incubated with PBS containing 0.5% Nonidet P-40. Coverslips were incubated with 1:100 dilution of the appropriate primary antibody for 1 h at room temperature. After three rinses with PBS containing 0.5% Tween 20, coverslips were incubated for 1 h at room temperature with 1:100 dilution of Texas Red- or fluorescein isothiocyanate-conjugated secondary antibody (Jackson ImmunoResearch Laboratories). Coverslips were then rinsed three times with PBS containing 0.5% Tween 20 and mounted onto glass slides. Fluorescent signals were detected by using either epifluorescence or confocal microscopy.

FRET. U2-OS cells were grown and cotransfected with the appropriate YFP- and CFP-tagged expression constructs in 35-mm uncoated glass bottom cell culture dishes (MatTek). Live cell images were obtained with a Zeiss LSM510 Meta laser scanning confocal microscope with a Plan- Apochromat × 63 objective lens and a 30-mW Argon laser set at 50% of total output. CFP as the donor channel was excited with a 458-nm laserline, and CFP fluorescence was collected with a band-pass filter of 475 to 525 nm. YFP, the acceptor channel, was excited at 514 nm, and YFP emission was collected with a long-pass filter of 530 nm. The
fluorescence resonance energy transfer (FRET) channel consisted of CFP excited at 458 nm and YFP fluorescence collected with a long-pass filter of 530 nm. Photobleaching was performed with the 514-nm laser line set at 100% power with an average bleach time of 5 s. Specific regions of interest (ROIs) were chosen, and positive FRET was determined graphically based on the decrease of YFP signal, and the subsequent increase in the CFP fluorescence postbleaching. Although transfection of any combination of YFP-RPA2 and CFP-nucleolin (or nucleolin derivative) did not have notable deleterious effects on cell viability, only cells with a normal appearance and relatively low expression levels were tested.

**Results**

**Genotoxic stress induces RPA-nucleolin complex formation.** We previously showed that heat shock led to a significant increase in complex formation between endogenous nucleolin and RPA (13). We therefore determined whether this increase was specific for heat shock or a more general effect in response to stress. Human U2-OS osteosarcoma cells were treated with the radiomimetic agent camptothecin (CPT) to cause genotoxic stress (Fig. 1A). Although RPA-nucleolin complex formation was not found in control cells, these complexes were readily detected after CPT treatment with a transient increase in the level of complex formation noted. We estimate that 5 to 10% of the RPA pool is coimmunoprecipitated with nucleolin at the peak level of complex formation, although this value would be an underestimate if the complex were transient or unstable under immunoprecipitation conditions. A similar induction of nucleolin-RPA complex formation was observed in p53-null H1299 cells after CPT treatment (Fig. 1E). Therefore, although nucleolin relocalization from the nucleolus to the nucleoplasm is p53 dependent (14), this dependence does not extend to nucleolin-RPA complex formation. Note that previous studies from our laboratory indicated that complex formation is not mediated by the presence of DNA and can also be detected by precipitation of RPA rather than nucleolin (13). In general, enhanced nucleolin-RPA complex formation is not restricted to heat shock but is also detected after genotoxic stress.

**RPA interacts with the nucleolin GAR domain in vitro.** To better characterize the nucleolin-RPA complex, the region on human nucleolin that interacts with RPA was identified by far-Western analysis. Full-length nucleolin or nucleolin truncation mutants were expressed as GST-tagged fusion proteins in yeast and purified. The proteins tested were full-length nucleolin (termed nucleolin FL; aa 1 to 707), the nucleolin N terminus (NT; aa 1 to 323), the C terminus (CT; aa 323 to 707), the N terminus and the first RBD (NT/RBD1; aa 1 to 390), the N terminus and the RBD region (NT/RBD1-4; aa 1 to 648), and the extreme C-terminal GAR domain (GAR; aa 645 to 707) (Fig. 2A). Note that the NT/RBD1-4 construct lacks only
the GAR region. After SDS-PAGE and transfer to a nitrocellulose membrane, the immobilized proteins were renatured and incubated with purified RPA to allow for complex formation. The interaction between the fusion proteins and RPA was resolved by Western blotting with an RPA2 antibody (Fig. 2B, upper panel).

Nucleolin FL formed a complex with RPA (Fig. 2B, upper panel, lane 2), whereas GST alone did not (lane 1). Although no interaction with nucleolin NT was detected (lane 3), RPA bound to the C-terminal half of the protein (lane 4). Longer constructs of nucleolin NT that also contained the first RBD (NT/RBD1; lane 5) or the complete RBD domain (NT/RBD1-4; lane 6) were unable to rescue nucleolin-RPA complex formation. In contrast, RPA effectively bound the 63-aa GAR peptide lacking all other nucleolin domains. Stripping the blot and reprobing the membrane with anti-GST antibodies indicated that similar amounts of each GST fusion protein were loaded on the membrane (Fig. 2B, lower panel). These data demonstrate that the nucleolin GAR domain is necessary and sufficient for RPA binding in vitro. A fraction of each GST construct was invariably present in a degraded form but only the largest FL, CT, or GAR species was observed to bind RPA. The GST constructs are degraded from the C-terminal end because N-terminal deletions would prevent reactivity to the anti-GST antibody (i.e., the GST is located on the N terminus of each construct). We therefore suggest that the extreme C terminus of the GAR domain is required for significant RPA binding.

Effect of the GAR domain on SV40 DNA replication in vitro. We previously showed that SV40 DNA replication in vitro was inhibited by the addition of nucleolin, purified from human cells, which interfered with RPA action (13). Because our data indicate that the nucleolin GAR domain interacts with RPA, we similarly tested the effect of this peptide on SV40 DNA replication. GST-tagged nucleolin or nucleolin derivatives were purified, and titrated into a T-antigen-dependent SV40

FIG. 2. The nucleolin RPA-binding domain inhibits SV40 DNA replication in vitro. (A) Schematic showing GST-tagged nucleolin and nucleolin mutant proteins as follows: full length (FL), amino terminus (NT), carboxy terminus (CT), amino terminus including the first RBD (NT/RBD1), the GAR deletion mutant (NT/RBD1-4), and only the C-terminal GAR domain (GAR). For each construct, the N-terminal acidic domain is indicated in dark gray; each of the four RBDs have light gray shading and are numbered, and the GAR domain is shown in black. (B) Far-Western analysis of the nucleolin-RPA interaction. Equivalent amounts (500 ng) of nucleolin FL (lane 2), NT (lane 3), CT (lane 4), NT/RBD1 (lane 5), NT/RBD1-4 (lane 6), and GAR (lane 7), with each containing an N-terminal GST tag, were separated by SDS-PAGE. GST alone was also electrophoresed as a control (lane 1). After transfer to a nitrocellulose membrane, the membrane was probed with purified RPA (0.2 μg/ml) (upper panel, lanes 1 to 7). The binding of RPA was visualized by using an RPA2 antibody. To visualize GST-tagged proteins, the membrane was stripped and subjected to immunoblot analysis with a rabbit anti-GST antibody (lower panel, lanes 1 to 7). (C) An SV40 ori-containing plasmid (180 ng) was incubated with AS65 extract (100 μg), T antigen (750 ng), RPA (200 ng), and purified GST-tagged nucleolin proteins (as indicated) for 2 h at 37°C (52). Both FL (○) and GAR (×) GST-tagged nucleolin proteins are proficient in inhibiting SV40 DNA replication in vitro, whereas the NT/RBD1-4 (△) GST-tagged nucleolin protein and GST alone (○) are not. Replication activity was determined by precipitating the reaction mixtures with trichloroacetic acid and determining the amount of 32P in the precipitate by scintillation counting. The data was plotted as the relative DNA replication inhibition compared to that determined by using 100 ng of GST-FL. The maximum degree of inhibition was to 68% that of control levels.
DNA replication reaction (Fig. 2C). In reactions containing nucleolin FL or GAR, DNA synthesis was significantly inhibited as a function of the amount of nucleolin protein added. In contrast, no obvious inhibition was seen by addition of nucleolin NT/RBD1-4 or GST. Thus, nucleolin molecules that are capable of binding RPA also inhibit DNA replication in vitro.

**Stress-dependent formation of the nucleolin FL-RPA complex.** We examined the interaction of nucleolin and the nucleolin mutants with RPA in vivo. Because the cellular localization of nucleolin may be a determinant affecting its interaction with RPA (13), we first examined the localization of the different nucleolin derivatives. GFP-tagged nucleolin FL, NT/RBD1-4, and GAR were expressed in U2-OS cells, and the localization of the fusion proteins captured by indirect immunofluorescence microscopy. Nucleolin FL localized exclusively to nucleolar regions (Fig. 3B), as determined by colocalization with endogenous nucleolin and upstream binding factor (necessary for RNA polymerase I-mediated transcription of rRNA [27]) (data not shown). The NT/RBD1-4 and GAR proteins had primary localization in the nucleolus (Fig. 3C and D, respectively), although the level of nucleolar staining was higher for the NT/RBD1-4 mutant. A significant fraction of each mutant protein pool was located in the nucleoplasm, and both mutants showed a weak but clear cytoplasmic signal. As expected, GFP alone was localized throughout the cell (Fig. 3A). These data are consistent with previous findings that the nucleolin RBD and GAR domains each contribute to nucleolar localization (12, 28, 40, 47).

The ability of various GFP-tagged nucleolin proteins to associate with endogenous RPA in vivo was tested by coimmunoprecipitation assays. RPA coprecipitated with the GAR domain but did not associate with the NT/RBD1-4 mutant (Fig. 3I, lanes 8 and 7, respectively). Nucleolin FL did not significantly complex with RPA under these nonstress conditions (lane 6) although, because of the higher background in the upper regions of the blot, we cannot rule out a low level of complex formation. To rule out the possibility that the large GFP moiety may sterically block nucleolin complex formation with RPA, reverse immunoprecipitation experiments were repeated with nucleolin tagged with a smaller Myc tag (Fig. 3J). Test of the Myc-tagged nucleolin proteins showed that only nucleolin GAR formed detectable complexes with endogenous RPA (lane 6), whereas nucleolin FL (lane 4) and NT/RBD1-4 (lane 5) did not. Note that detection of Myc-GAR in cell lysates by Western blotting was problematic because of poor association of this 5-kDa species with the nitrocellulose mem-
that contains the GAR domain coprecipitated with RPA both irrespective of stress (Fig. 4C, middle panel). The CT construct the GAR domain did not form a detectable complex with RPA to associate with nucleolin FL but only after CPT treatment nucleoplasm (Fig. 4B), similar to the behavior of endogenous a significant fraction of the nucleolin FL pool to move to the (Fig. 4A [see also Fig. 3B above]), incubation with CPT caused nucleolin FL localized to the nucleolus in the absence of stress (50, 52). In cells trans-

brane. However, the levels of Myc-tagged nucleolin FL, NT/ RBD1-4, and GAR were comparable when examined in parallel experiments by immunofluorescence microscopy, and their cellular localizations were similar to those observed for the analogous GFP fusion proteins (data not shown). In sum, these data indicate that the nucleolin GAR domain is sufficient to support complex formation with RPA in vivo. Concerning the lack of association between nucleolin FL and RPA in vivo, although in apparent contradiction with the results of the Far Western analysis in vitro (above), these results are consistent with those showing a lack of complex formation between endogenous nucleolin and RPA in nonstressed cells (see Fig. 1, zero time points).

We next examined the effect of CPT treatment on GFP-tagged nucleolin FL localization and on the interaction of RPA with nucleolin FL and the nucleolin derivatives. Although nucleolin FL localized to the nucleolus in the absence of stress (Fig. 4A [see also Fig. 3B above]), incubation with CPT caused a significant fraction of the nucleolin FL pool to move to the nucleoplasm (Fig. 4B), similar to the behavior of endogenous nucleolin (13). In testing the interactions, RPA was observed to associate with nucleolin FL but only after CPT treatment (Fig. 4C, upper panel). In contrast, the NT construct lacking the GAR domain did not form a detectable complex with RPA irrespective of stress (Fig. 4C, middle panel). The CT construct that contains the GAR domain coprecipitated with RPA both in the presence of CPT and in its absence, thus revealing a constitutive interaction (Fig. 4C, lower panel). The localizations of these truncated proteins were not affected by prior CPT treatment (data not shown). These data indicate that although the presence of the GAR is necessary to support detectable complex formation with RPA in vivo, detectable interaction of RPA with the full-length nucleolin also requires stress conditions such as caused by CPT treatment.

Nucleolin TM is able to mimic endogenous nucleolin under conditions of stress. To examine the question of whether nucleolin localization regulates nucleolin-RPA complex formation in vivo, we generated a nucleolin mutant with altered cellular localization. Preliminary studies by our laboratory indicate that the nucleolin phosphorylation pattern at CK2 sites changes in response to stress (K. Kim, M. Daras, and J. A. Borowiec, unpublished data). The three putative CK2 sites at positions S33, S187, and S209 were therefore converted to nonphosphorylatable alanines to generate nucleolin TM (for triple mutant. The localization of GFP-tagged nucleolin TM was examined in untreated U2-OS cells or in cells treated with CPT. Interestingly, nucleolin TM was found to have significant localization in the nucleoplasm in the absence of DNA damage (Fig. 5C) and resembled the localization of nucleolin FL in cells treated with CPT (Fig. 5B). After exposure of the cells to CPT, nucleolin TM was seen to have an even greater fraction of signal arising from the nucleolus (Fig. 5D). In testing interactions, the coprecipitation of nucleolin TM with RPA was found to be constitutive and independent of prior CPT treatment (Fig. 5E, lanes 3 and 4), in contrast to nucleolin FL (lanes 1 and 2). Therefore, a nucleolin mutant with a significant degree of nucleoplasmic localization in nonstressed cells also has a constitutive interaction with RPA.

Nucleolin-RPA complex formation examined by FRET. To examine whether a nucleoplasmic localization of nucleolin assists complex formation with RPA, we used FRET to determine the cellular site(s) of interaction. The middle subunit of heterotrimeric RPA (RPA2) was expressed as a YFP fusion, whereas nucleolin and the nucleolin derivatives were coupled to CFP. Previous studies testing GFP-RPA2 indicate that it behaves similarly to the endogenous RPA2 subunit, including the association with replication centers (50, 52). In cells transfected with both YFP-RPA2 and CFP-nucleolin FL, CPT treatment caused nucleolin relocalization (Fig. 6D) as seen above while having little notable affect on YFP-RPA2 (compare Fig. 6B and E). Although no significant FRET signal was detected in the absence of CPT treatment (Fig. 6C), a robust FRET signal was seen when these same doubly transfected cells were analyzed after CPT treatment (Fig. 6F). Because FRET is subject to artifactual detection due to CFP signal bleedthrough into the YFP channel, we performed acceptor photobleaching in which bleach of the YFP fluorescence stimulates the emission from CFP (Fig. 6G) (33). Cells that were either mock treated or treated with CPT were analyzed by using ROIs located in either the nucleolus or the nucleoplasm, and the average CFP signals from these experiments is shown. Consistent with the FRET images, no significant photobleach-dependent stimulation of the CFP signal was observed either in the nucleolus or the nucleoplasm without CPT. When cells were treated with CPT, a robust increase in the CFP signal was detected in the nucleoplasm and the nucleolus after photo-
bleaching. These FRET signals were quantitated and normalized against that found by nucleolin FL and RPA in the nucleoplasm after CPT treatment (Table 1).

We next performed similar FRET analyses with the nucleolin GAR and NT/RBD1-4 domains. Both nonstressed cells and cells treated with CPT were examined. The average normalized change in the CFP signal after YFP photobleaching was provided (Table 1). From these data, we found that the GAR domain of nucleolin interacts with RPA irrespective of the presence of CPT and equally well in the nucleolus and the nucleoplasm. Similarly, the nucleolin TM mutant showed a very strong FRET signal in both the nucleolus and the nucleoplasm in a CPT-independent fashion. In contrast, the NT-RBD construct was not found to interact with RPA in either the nucleolus or the nucleoplasm and without apparent effect from the CPT. We note that these latter data are subject to the standard concerns of false-negative FRET results due to potential improper orientation of the two fluorescent tags. As a positive control, we show a significant FRET signal from the H-Ras 61L with the Ras-binding domain of Raf1 in the cytoplasm (4). As expected, no FRET signal arises from cells expressing the H-Ras 61L and RPA2 or in cells expressing CFP-nucleolin alone. We also show that heat shock induces a stronger FRET signal compared to CPT treatment, which parallels our previous immunoprecipitation findings that heat shock also greatly stimulated the nucleolin-RPA complex formation (13). It is interesting that heat shock also has a much greater effect on chromosomal DNA replication (an approximately 70 to 85% reduction [see, for example, references 13 and 55]) compared to genotoxic stress (an approximately 50% reduction [see, for example, reference 42]). In sum, along with confirming that the nucleolin FL-RPA interaction is stress dependent, these data also indicate that nucleolin-RPA complex formation is stimulated in both the nucleolus and the nucleoplasm after genotoxic stress.

FIG. 5. The nucleolin TM mutant constitutively interacts with endogenous RPA. The subcellular localization of nucleolin FL (A and B) and nucleolin TM (C and D) in U2-OS cells was determined. At 24 h posttransfection, cells were either mock treated (A and C) or examined 2 h after treatment with 1 μM CPT for 1 h (B and D). Cells were prepared for epifluorescence microscopy as described in Materials and Methods. (E) The coprecipitation of Myc-tagged nucleolin FL and TM with endogenous RPA in U2-OS cells was examined 24 h posttransfection either with or without prior CPT treatment (as described above). Endogenous RPA was precipitated with anti-RPA2 antibody, and the coprecipitation of Myc-tagged nucleolin FL or TM was visualized by Western blotting with an anti-Myc antibody (9E10).

FIG. 6. Nucleolin FL-RPA complex formation occurs both in the nucleolus and the nucleoplasm after stress. (A to F) U2-OS cells were transfected with CFP-nucleolin and YFP-RPA2 and either mock treated or treated with 1 μM CPT for 1.5 h prior to imaging. Cells were then imaged to capture the CFP-nucleolin signal (A and D), the YFP-RPA2 signal (B and E), or the FRET signal obtained by transfer of the CFP emission energy to YFP (C and F). The FRET images are shown with a pseudo three-dimensional display with the intensity of staining given on the z axis. (G) Acceptor photobleaching analysis of nucleolin-RPA complex formation. The CFP signal from various (ca. 10 to 15) ROIs was determined at 6-s intervals. After the fifth scan, the YFP fluor was photobleached at 514 nm with an average bleach time of 5 s. An increase in the CFP after photobleaching of the YFP signal is indicative of bona fide FRET (33).
Cell cycle arrest upon overexpression with either nucleolin GAR or TM. We found previously that heat shock mobilizes nucleolin to move to the nucleoplasm, whereupon it binds RPA at sites distinct from the DNA replication centers (13). These data predict that expression of nucleolin derivatives that bind RPA in nonstressed cells will cause a G1/S arrest. The effect of nucleolin TM and GAR expression on cell cycle progression were therefore investigated by FACS. Both the nontransfected control and the vector control showed a similar distribution, indicating that transfection alone did not inhibit cell cycle transit (Fig. 7A). Expression of nucleolin FL led to only a slight increase in G1-phase cells. However, much more significant effects were observed in cells transfected with nucleolin TM or GAR. The expression of nucleolin FL or GAR resulted in an increase in the G1 population to 52% of cells compared to 36% of vector-transfected cells. We also detected a decrease in S-phase cells from 39% in vector-transfected cells to 23% in GAR-transfected cells. Expression of nucleolin TM had a similar influence on cell cycle progression with G1- and S-phase cells contributing 53 and 25%, respectively, of the total cell pool. In each case, the fraction of G2 cells remained constant. Thus, the expression of nucleolin GAR or TM was sufficient to elicit an arrest in the cell cycle, leading to the accumulation of cells in G1 and a decrease in cells in S phase. We note that the overall degree of replication inhibition in the GAR- or TM-transfected cells (a >40% decrease) is similar to that observed after ionizing irradiation (42).

DNA replication inhibition overcome by overexpression of RPA2. If the inhibition of DNA replication by nucleolin GAR or TM were truly mediated through RPA, then overexpression of heterotrimeric RPA might overcome this inhibition of DNA synthesis. A method of increasing RPA levels arose from our finding that changes in RPA2 levels have coordinate effects on the level of the RPA1 subunit. That is, a decrease in RPA2 levels due to the use of RNAi leads to a corresponding decrease in RPA1 levels (D. Curanovic and J. A. Borowiec, unpublished results), a finding also recently reported by others (16). Similarly, overexpression of Myc-RPA2 caused a parallel increase in the level of RPA1 protein, when examined either by Western blotting (Fig. 7B) or immunofluorescence microscopy (data not shown). Since stable association of the RPA1 and RPA2 subunits requires the smallest RPA3 subunit (which available antibodies only poorly detect), these data indicate that changes in the level of RPA2 regulate the level of RPA in cells. Overexpression of RPA2 thereby provides a method to more directly examine the nucleolin-RPA interplay in inhibiting chromosomal DNA replication.

To test this method, U2-OS cells were transfected with either nucleolin GAR or TM, and the level of DNA replication was measured by determining thymidine incorporation (Fig. 7C). Corroborating the results of the FACS analysis, expression of either nucleolin construct inhibited DNA synthesis by ca. 50%. In parallel reactions, these cells were cotransfected with increasing levels of RPA2. We observed that the degree of replication inhibition caused either by nucleolin GAR or TM expression was progressively reduced by transfection of the Myc-RPA2 expression vector. The stimulatory effect of RPA2 overexpression was somewhat more pronounced in nucleolin TM-transfected cells compared to GAR-transfected cells, for unknown reasons. Transfecting higher levels of RPA2 vector (i.e., 100 ng) caused some toxic effects on cell viability (data not shown). These data strongly indicate that nucleolin can inhibit DNA synthesis by direct interaction with RPA.

Nucleolin GAR expression does not activate p53. It is possible that the expression of the GAR domain causes a cellular stress response and therefore only inhibits cell cycle progres-
As a test of this possibility, we examined the effect of GAR expression on p53 activation in U2-OS cells (which express wild-type p53). Expression of nucleolin GAR did not increase p53 levels (Fig. 7D, upper panel) or the level of p53 phosphorylated on Ser15, a site modified by the ATM and ATR kinases in response to genotoxic stress (49, 51). The lack of Ser15 phosphorylation demonstrates that p53 and, indirectly, ATM and ATR do not become activated in response to GAR expression (Fig. 7D, second panel). Aliquots of these lysates were probed for the presence of p21waf1, a key stress-induced inhibitor of cyclin-dependent kinases whose expression results in a G1/S arrest. No changes in the level of p21waf1 were detected in response to GAR expression (Fig. 7D, third panel). In contrast, treatment of cells with CPT was found to simultaneously stimulate the levels of p53, (pSer15)p53, and p21waf1. The block in cell cycle progression caused by expression of nucleolin GAR is therefore unrelated to p53 activation, induction of p21, or, likely, activation of ATM or ATR. Instead, our data indicate that nucleolin can itself inhibit DNA replication by binding to RPA and inhibiting RPA activity.

**DISCUSSION**

In response to genotoxic insult and other stress conditions, eukaryotic cells in S phase use multiple mechanisms to reduce the level of ongoing DNA replication and thereby minimize the detrimental repercussions to the genome. Certain stress response pathways inhibit S-phase kinase complexes Cdk2/cyclin E (10, 19) and Cdc7/Dbf4 (11) whose activities are necessary to allow an origin of replication to fire. Another route apparently mediating the S-phase checkpoint targets the Mre11 recombinational DNA repair complex (43). In contrast, the pathway that we identify involves the inhibition of an essential DNA replication factor, RPA, by stress-dependent complex formation with nucleolin. In this pathway, our data indicate that nucleolin becomes activated in response to stress,
leading to heightened complex formation in both the nucleolus and the nucleoplasm. This induced nucleolin-RPA complex can block cellular transit through the G$_{1}$/S boundary and inhibit DNA replication during S phase. Furthermore, the nucleolin-mediated inhibition can be diminished by heightened expression of RPA.

What is the mechanism by which nucleolin inhibits cell cycle progression? We find that GST-tagged nucleolin FL and the GAR domain each can inhibit SV40 DNA replication in vitro, recapitulating similar inhibitory effects that were observed when endogenous nucleolin purified from human cells was tested (13). Although our studies did not find an inhibitory effect of nucleolin on RPA binding to single-stranded DNA, we do find that nucleolin can inhibit the binding of RPA to duplex molecules containing a central nonpaired region (data not shown). Such data suggest that the nucleolin-RPA complex is selectively inhibitory to the initiation stages of replication. However, we have recently presented data indicating that RPA does not randomly bind to single-stranded DNA at a chromosomal DNA replication fork but is instead actively loaded by a component of the replication machinery (52). Thus, complex formation with nucleolin has the potential to prevent RPA from productive loading onto single-stranded DNA at a replication fork in vivo. Both of these processes could inhibit DNA replication in vivo and cause a reduction in DNA synthesis. Overall, our data indicate that a direct interaction between the GAR domain of nucleolin and RPA is sufficient for replication inhibition in vivo.

Our data lead to the model that genotoxic stress activates nucleolin, such that the GAR domain becomes exposed for complex formation with RPA. In support of this model, consider the following data. First, although the GAR domain is required to bind RPA, nucleolin FL also requires stress conditions to bind RPA. Second, the nucleolin TM molecule that constitutively binds RPA was mutated at three N-terminal positions, whereas the RPA-interacting GAR domain is located on the extreme C terminus of nucleolin. Third, nucleolin relocalization to the nucleoplasm, although an outcome of genotoxic stress and heat shock, is not required for RPA complex formation because our FRET data show interaction in the nucleolus, as well as in the nucleoplasm. Fourth, a requirement for changes in RPA modification does not appear to be required as the GAR domain binds RPA in a stress-independent fashion. Along these same lines, preliminary evidence obtained from test of a hyperphosphorylation mimic of RPA (RPA$_{2p}$) (52) showed no significant effects on nucleolin complex formation compared to RPA$_{2p}$ (data not shown). We postulate that changes in nucleolin modification promote conformational changes which remove steric constraints preventing RPA complex formation. Although expression of nucleolin TM or GAR do not cause apparent ATM or ATR activation, it is quite possible that activation of these kinases by genotoxic stress facilitates nucleolin-RPA complex formation, a possibility under investigation.

Our FRET data indicate that nucleolin-RPA complex formation occurs both in the nucleolus and in the nucleolus and hence nucleolin relocalization is not required for these two proteins to interact. Even so, nucleolin relocalization probably facilitates interaction with RPA. The nucleolus comprises ca. 10 to 15% of the nuclear volume in human cells (e.g., see reference 18) and a nucleoplasmic localization would provide a larger volume in which complex formation can occur. Although p53 is not required for nucleolin-RPA complex formation, nucleolin relocalization is strongly dependent on p53 (14) (see also below), suggesting that p53 might stimulate the nucleolin-RPA interaction. Testing the ability of p53-positive (U2-OS) and negative (H1299) cells to induce nucleolin-RPA complex formation after stress did not reveal any obvious differences. That said, these cells have genetic differences other than p53 that preclude our drawing firm conclusions on the potential role of p53 in facilitating complex formation at this time.

The mechanism of nucleolin relocalization remains somewhat unclear. Previous study has found that movement of a portion of the nucleolin pool to the nucleoplasm is greatly facilitated by p53 (14). Because genotoxic stress transiently induces nucleolin and p53 complex formation (14), increased nucleoplasmic levels of appropriately modified p53 and nucleolin may lead to more complex formation and hence a net nucleoplasmic flow of nucleolin. The lack of requirement for p53 in supporting nucleolin-RPA complex formation would indicate that an event(s), such as changes to the nucleolin modification state, occurs prior to nucleolin-RPA and nucleolin-p53 complex formation. This event would lead to the apparently independent increase in the association of nucleolin with either p53 or RPA. Along with a p53 requirement in supporting nucleolin mobilization from the nucleolus in response to stress, it has also been recently proposed that p53 activation by stress itself involves nucleolar disruption (ND) (46). In this model, ND interrupts a requisite nucleolar export pathway for p53 destined for degradation. If this model is correct, ND initiates p53 activation, which itself leads to increased ND.

We identified the nucleolin GAR domain as being necessary for interaction with RPA in vitro and in vivo. The GAR domain is contained within ~65 residues and includes more than 10 RGG or FGG repeats. Similar RGG/FGG repeat sequences are found in other RNA-binding proteins, including hnRNP A1, hnRNP U, and fibrillarin (3). The RGG region forms a β-spiral structure and binds nonspecifically to single- and double-stranded RNA and DNA (21). The GAR domain of nucleolin interacts with various ribosomal subunits, including L3 (22), and, along with its ability to bind RNA, presumably explains the role of the nucleolin GAR domain in supporting efficient nucleolar localization (12, 28, 40, 47). The nucleolin GAR domain also contains a 12-residue unique lysine-rich element at the extreme C terminus. Our far-Western analysis indicates that nucleolin molecules with small C-terminal deletions do not support RPA binding, suggesting that RPA may bind this unique C-terminal end. Additional studies will be needed to determine the relative contributions of the RGG region and the unique element in supporting complex formation with RPA.

It is becoming clear that the nucleolus is a critical cellular body whose components regulate cell cycle progression. For example, p19$^{ARF}$ (p14$^{ARF}$ in humans) localizes to the nucleolus, where it can bind and sequester the p53 antagonist MDM2 and thereby cause p53 stabilization (56). Similarly, the binding of the human MDM2 RING domain to ATP stimulates nucleolar localization in the absence of p14$^{ARF}$ (44). The yeast Yph1p protein is a BRCT domain-containing nucleolar factor
whose depletion causes both G, and G2 arrest (17). With regard to mitotic progression, it has been found that exit from mitosis is controlled by the Cdc14 protein phosphatase that is sequestered in the nucleolus until anaphase (2). These and other observations, combined with our findings that nucleolus also serves a dual role in ribosome biogenesis and inhibiting S-phase progression in response to genotoxic stress, highlights the importance of the nucleolus in serving to integrate cell growth and cell stress pathways.

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Replication Protein A (RPA) Phosphorylation Prevents RPA Association with Replication Centers

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Mammalian replication protein A (RPA) undergoes DNA damage-dependent phosphorylation at numerous sites on the N terminus of the RPA2 subunit. To understand the functional significance of RPA phosphorylation, we expressed RPA2 variants in which the phosphorylation sites were converted to aspartate (RPA2D) or alanine (RPA2A). Although RPA2A was incorporated into RPA heterotrimers and supported simian virus 40 DNA replication in vitro, the RPA2D mutant was selectively unable to associate with replication centers in vivo. In cells containing greatly reduced levels of endogenous RPA2, RPA2D again did not localize to replication sites, indicating that the defect in supporting chromosomal DNA replication is not due to competition with the wild-type protein. Use of phosphospecific antibodies demonstrated that endogenous hyperphosphorylated RPA and RPA2A associates with DNA damage foci as determined by colocalization with γ-H2AX. We conclude that RPA2 phosphorylation prevents RPA association with replication centers in vivo and potentially serves as a marker for sites of DNA damage.

DNA-damaging stress leads to the inception of a variety of cellular responses that serve to minimize mutation and prevent genomic instability. In particular, the cell cycle checkpoint apparatus is activated to block S phase entry and, in those cells in the replicative phase, to both inhibit firing of late origins of DNA replication and arrest the collapse of replication forks blocked by damage (3). The DNA repair machinery is mobilized in concert to repair lesions and to allow eventual restart of stalled replication forks. One factor that plays essential roles both during DNA replication and in the repair- and recombination-mediated recovery from damage is replication protein A (RPA), the eukaryotic single-stranded (ss) DNA-binding protein (27, 52).

RPA is a heterotrimeric protein consisting, in mammalian cells, of ~70- (RPA1), 30- (RPA2), and 14 (RPA3)-kDa subunits. During DNA replication, RPA acts at the fork, stabilizing ssDNA and facilitating nascent strand synthesis by the replicative DNA polymerases. Under DNA-damaging conditions, RPA-ssDNA complexes act to recruit and activate a key checkpoint mediator consisting of the ATR and ATRIP (ATR-interacting protein)-protein-kinase complex (54). At DNA damage-dependent nuclear foci, RPA interacts with repair and recombination components to process double-strand DNA breaks and other lesions (19). RPA activity is regulated by various stress conditions. In particular, heat shock (12, 47, 48), exposure to UV radiation (9), and treatment with DNA-alkylating agents (30) each cause the generation of an RPA species that is unable to support DNA replication in vitro. In the case of heat shock, the inhibition of RPA activity is mediated by a stress-dependent association with the nucleolar protein nucleolin (12, 47).

In an area with potential regulatory significance, RPA undergoes both stress-dependent and -independent phosphorylation on the extreme N terminus of the RPA2 subunit. A basal level of RPA modification by cyclin-cdk complexes occurs at two sites (16, 35). Following stress, such as exposure to ionizing (31) or UV (9) radiation, or treatment with radiomimetic agents, such as camptothecin (CPT) (42), human RPA2 can be phosphorylated at five or more additional sites out of a possible seven by the phosphatidylinositol 3-kinase-related kinases (PIKKs) DNA-PK, ATM, and perhaps ATR (7, 10, 17, 18, 31, 33, 35, 46, 53). ATM and ATR are activated in response to DNA damage and replication stress, and they modify various effectors that facilitate the damage and cell cycle checkpoint responses (1). DNA-PK is required directly in the repair of double-strand DNA breaks and in V(D)J recombination (15). These data could suggest that the function of stress-dependent modification of RPA is to repress DNA replication or to promote recovery from DNA damage, but there are as yet no compelling data for either role. While the results of certain studies suggest that RPA modification by PIKKs may lead to the inhibition of DNA replication in vitro and in vivo (9, 37), direct testing of this possibility has not shown any appreciable effects of RPA phosphorylation on binding to ssDNA or on replication in vitro using a simian virus 40 (SV40)-based assay (7, 23).

Because previous work has primarily studied the effects of mammalian RPA phosphorylation using in vitro systems, it is possible that the modulation of RPA activity by phosphorylation might be observed only in the cellular milieu. Testing this hypothesis, we found that RPA2 phosphorylation mutants that mimic the hyperphosphorylated form were unable to localize to replication centers in normal cells. Interestingly, binding of
the hyperphosphorylation mimic to DNA damage foci was unaffected, as determined by colocalization with the DNA damage marker γ-H2AX. Similar behavior was observed with endogenous hyperphosphorylated RPA. We conclude that RPA phosphorylation following damage both prevents RPA from catalyzing DNA replication and potentially serves as a marker to recruit repair factors to sites of DNA damage.

**MATERIALS AND METHODS**

**Cell lines and stress treatments.** U2-OS and HeLa cells were maintained in McCoy’s 5 M and Dulbecco’s modified Eagle’s media, respectively, supplemented with 10% fetal bovine serum and 50 μg of gentamicin/ml. When the effect of stress was examined, the cells were treated with either 1 μM CPT (Sigma) for 1 or 3 h, 2.5 mM hydroxyurea (HU; Sigma) for either 1 or 3 h, or 7 μM aphidicolin (Sigma) for 3 h. To inhibit the cellular checkpoint response, cells were treated with 5 mM caffeine for 30 min prior to stress. Transfection experiments were performed using Effectene (Qiagen).

**Generation of RPA2 mutant constructs.** To generate the myc-RPA2wt and myc-RPA2D mammalian expression vectors, the human RPA2 genes from plasmids p11drRPA and p11drRPA-32Asp (4, 24) were inserted into the Bst and BstBI sites of the pEF6/Myc-HisA vector (Invitrogen), resulting in pERPA2wt and pERPA2D. Expression of the His6 tag from pEF6/Myc-HisA was prevented by mutating the ATG codon at position 1863 to a TGA codon. Vectors expressing the intermediate RPA2 phosphorylation mutants and RPA2wt were constructed by a combination of site-directed mutagenesis of either pERPA2wt or pERPA2D (as appropriate) at positions 23, 29, and 33 and replacement of larger segments of the RPA2 N terminus with synthetic oligonucleotides encoding mutant phosphorylation regions. Detailed cloning procedures are available upon request.

**Protein purification and in vitro replication assay.** The RPAwt and RPA mutants were expressed in Escherichia coli BL21 transformed with p11drRPA and p11drRPA-32Asp, respectively, and purified as described previously (24, 26). The SV40 large tumor (T) antigen used for SV40 DNA replication reactions was prepared from extracts of Sf9 cells infected with the baculovirus US7 (24, 27). SV40 DNA replication reaction mixtures (50 μl) containing 40 μM creatine phosphate (dTri salt; pH 7.8); 7 mM MgCl₂; 4 mM ATP; 250 M (each) CTP, GTP, and UTP; 100 μM (each) dATP, dGTP, and dCTP; 25 μM [32P]dCTP (1,000 cpm/pmol); 0.2 μg of the ori-containing plasmid pSV1AEP (50); 200 μg of the AS65 fraction; 0 to 700 ng of RPAwt or RPA2, and 750 ng of SV40 T antigen were incubated at 37°C for 2 h. Replication activity was determined by precipitating the high-molecular-weight DNA with trichloroacetic acid and quantitating the amount of [3H]dCTP incorporated. To examine the DpnI resistance of the replication products, replication reaction mixtures containing 600 ng of either RPAwt or RPA2 and 100 μM [c-32P]dCTP (1,000 cpm/pmol) to label the replication products were incubated at 37°C for 2.5 h. Following removal of protein by phenol extraction, the DNA products were first linearized by digestion with PvuII and then either mock treated or incubated with 2.5 U of DpnI to cleave nonreplicated DNA. The digestion products were separated by electrophoresis through a 1.1% agarose gel and visualized both by ethidium bromide staining and by autoradiography.

**Immunoprecipitation and immunoblotting.** Transfected U2-OS cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% [vol/vol] NP-40, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM Na₃VO₃, 1 mM NaF, and 1 μg each of aprotinin, leupeptin, and pepstatin per ml). The cell extracts were then incubated at 4°C for 2 h with 70A anti-RPA1 monoclonal antibody (28) conjugated to CNBr-activated Sepharose beads (Amersham Biosciences). The immunoprecipitate was washed five times with lysis buffer and resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (15% [wt/vol] polyacrylamide). To test RPA2 phosphorylation and myc-RPA2 expression, cells were directly lysed in SDS-PAGE sample buffer and proteins were separated by SDS-PAGE. For phosphatase treatment, cells were lysed in λ protein phosphatase buffer (New England Biolabs) containing 1% Triton X-100 and 1 μg each of aprotinin, leupeptin, and pepstatin per ml. Cell lysates (20 μg of protein) were then incubated with 400 U of a protein phosphatase inhibitor (0.1 mM Na₃VO₃, 1 mM NaF). The Western blots were developed with an anti-RPA2 34A mouse monoclonal antibody (28) or a rabbit polyclonal anti-pSer4/pSer8-RPA2 antibody obtained from Bethyl Laboratories, Inc. (Montgomery, Tex.). Proteins were detected using enhanced chemiluminescence (Amersham Biosciences).

**Cell cycle assay.** Forty-eight hours posttransfection, cells were incubated with 10 μM bromodeoxyuridine (BrDU). After a 30-min incubation, the cells were fixed and processed according to the BrDU Flow Kit manual (BD Pharmingen). Following incubation with anti-BrdU (Harlan Sera-Lab) and rabbit anti-myC (Upstate Biotechnology) antibodies, the cells were stained with anti-rat fluorescein isothiocyanate-conjugated and anti-rabbit phycoerythrin-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories). The DNA was stained with 7-aminocanthinomycin D, and the cells were subjected to fluorescence-activated cell sorting (FACS) analysis.

**Immunofluorescence microscopy.** Transfected cells were processed by two methods. To test protein expression and transfection efficiency, the cells were first washed with phosphate-buffered saline (PBS), fixed with 4% [vol/vol] formaldehyde in PBS for 15 min at room temperature, and then extracted with PBS containing 0.5% (vol/vol) Triton X-100 for 5 min. To study chromatin-bound proteins, the cells were extracted with 0.5% (vol/vol) Triton X-100 for 5 min prior to fixation as described previously (13). When required, cells were incubated in media containing 10 μM BrdU for 10 min prior to harvest. For detection of incorporated BrdU, DNA was denatured with HCl using standard procedures. RPA2 silencing was achieved using a short interfering RNA (siRNA) duplex targeted to the 5'-CCUAGUUUCACAAACUACGUU sequence found in the 3' noncoding region of RPA2 mRNA. Prepared cells were incubated, as required, with rabbit anti-myC (Upstate Biotechnology), mouse anti-RPA1 70A and anti-RPA2 34A (28), rabbit anti-pSer4/pSer8-RPA2 (Bethyl Laboratories), rat anti-BrdU (Harlan Sera-Lab), and mouse anti-γH2AX (Upstate Biotechnology) antibodies. Following staining with the appropriate secondary antibodies (Jackson ImmunoResearch Laboratories), the cells were examined by epifluorescence microscopy using a Zeiss Axioskop microscope. To calculate the relative frequency of myc-RPA2-positive cells (see Fig. 6H and 8M), the fraction of cells transfected with myc-RPA2wt or the myc-tagged RPA mutants was first determined by processing cells without prior Triton X-100 extraction (e.g., Ftransfection/Ftransfection+D). Separately, the fraction of cells showing significant chromatin staining was also determined (e.g., Fchromatin/Dtransfection+D/Ftransfection+D × 100%). Each value determined was the result of three independent experiments.

**RESULTS**

The RPA2D phosphorylation mimic localizes to the nucleus but is not chromatin bound. To understand the functional significance of RPA phosphorylation, we generated various human RPA2 constructs in which subsets of the nine potential N-terminal phosphorylation sites were mutated. Previous studies have shown that two of the RPA2 sites (S23 and S29) are phosphorylated in a cell cycle-dependent manner by cyclin-cdk2 complexes (16, 35). At least five of the other seven (S4, S8, S11, S12, S13, T21, and S33) can be phosphorylated in response to UV irradiation (35). Ionizing irradiation and treatment with the radiomimetic agent CPT cause similar RPA hyperphosphorylation and likely modification of most if not all of these same sites (31, 42). Various data strongly suggest that the PIKK members DNA-PK and ATM, and likely ATR, can independently modify the RPA stress-dependent sites (7, 10, 17, 18, 31, 33, 35, 46), although only two (T21 and S33) have canonical SO/TQ sequences that are PIKK targets (1). Both of the cyclin-cdk2 sites and six of the stress-dependent sites (S8, S11, S12, S13, T21, and S33) were replaced by aspartate to mimic phosphate (generating the RPA2D mutant; see Fig. 6G for a schematic showing the construction of this and other mutants). Although an aspartate residue is not the same as phosphoserine or phosphothreonine, the use of aspartate residues to imitate phosphate has been shown in many cases to have identical effects on protein structure and activity (25, 49).
In the RPA2A mutant, these same eight sites were converted to alanines to prevent phosphorylation (see also Fig. 6G). All of the mutants and the wild-type RPA2 control (RPA2 wt) contained a C-terminal myc tag.

The RPA2wt subunit was expressed in human U2-OS cells. To detect the chromatin-bound fraction of RPA2, transfected cells were extracted with nonionic detergent prior to formaldehyde fixation (13). Under such conditions, RPA bound to chromatin in nuclear replication foci can be selectively visualized. The transfected RPA2wt subunit nearly completely colocalized with the endogenous RPA1 and exhibited a punctate distribution throughout the nucleus, consistent with its recruitment to DNA replication centers (Fig. 1A to D). To confirm this observation, transfected cells were pulse-labeled with BrdU prior to fixation, and the sites of RPA2wt localization and BrdU incorporation were examined. As expected, the RPA2wt subunit showed nearly complete colocalization with replicating chromatin (Fig. 1E to H). Taken together, these results indicate that the recombinant RPA2wt subunit can functionally replace endogenous RPA2 in supporting chromosomal DNA replication.

We next examined the localization of the RPA2A and RPA2D mutants. Transfected cells were examined both without and with prior detergent extraction to reveal transfection efficiency and to show the fraction bound to chromatin, respectively. The distribution of RPA2A on chromatin (Fig. 2L) was virtually identical to the replication pattern seen with endogenous RPA2 (data not shown) and the RPA2wt variant (Fig. 2D) and showed nearly complete colocalization with endogenous RPA1 (Fig. 2K and data not shown). RPA2 phosphorylation is therefore not required for association with replication centers.

In dramatic contrast, we did not observe chromatin staining for RPA2D (Fig. 2H), even though the RPA2D mutant was expressed to an equivalent similar to that of the RPA2wt construct (Fig. 2F and B, respectively). Similar experiments were performed with RPA2D and RPA2wt expressed as fusion proteins with green fluorescent protein. While a modest RPA2wt signal was detected, we did not observe an appreciable level of chromatin binding for RPA2D (data not shown). We therefore find that mutation of RPA2 to a hyperphosphorylation mimic greatly reduces its localization to DNA replication centers.

In addition to the possibility that phosphorylation of RPA inhibits its normal participation at the DNA replication fork in vivo, other explanations exist. One is that the myc-RPA2D subunit is unable to complex with the other RPA subunits. To examine this, lysates prepared from cells transfected with the RPA2wt and RPA2D expression vectors were subjected to immunoprecipitation with an anti-RPA1 antibody and immunoblotted for the presence of RPA2. The two myc-RPA2 variants, as well as the endogenous RPA2, efficiently coprecipitated with the RPA1 subunit (Fig. 3A, lanes 1 to 3). The RPA2D protein was also found in the lysate at levels comparable to those of RPA2wt, suggesting that the two proteins have similar stabilities (lanes 5 and 6). Because RPA1 and RPA2 complex formation requires the RPA3 subunit (24, 44), these data indicate that the two mutants form RPA heterotrimers with equivalent efficiencies.

To establish if heterotrimeric RPA containing the RPA2D subunit (RPA(RPA2D)) is inherently unable to function in DNA replication, we tested the abilities of RPA(RPA2D) and RPA2wt to support SV40 DNA replication in vitro. With the exception of the viral large T antigen, SV40 replication is
catalyzed by host cell components (8, 22). The SV40 system can thus provide a relatively comprehensive test of the ability of RPA to interact functionally with ssDNA and the DNA replication machinery. Previous work by J. Hurwitz and colleagues has shown that separation of human cell extracts by ammonium sulfate precipitation yields two required fractions (AS30 and AS65), with RPA found to be the only essential factor within the AS30 fraction (51). Because the AS65 fraction lacks RPA, the activities of different RPA variants can be assayed by their abilities to complement the AS65 fraction in supporting SV40 DNA replication. The RPARPA2D and RPARPA2wt variants were produced in E. coli and purified to homogeneity (Fig. 3B). Use of the AS65 fraction alone showed no significant DNA replication activity (Fig. 3C). The addition of either heterotrimeric RPA complex supported T-antigen-dependent viral DNA replication to similar extents, and the activities of the two RPA variants were similar over a range of levels (Fig. 3C). The reaction products synthesized in the presence of RPA_RPA2D or RPA_RPA2wt were equally resistant to DpnI, demonstrating that they were bona fide DNA replication products and not due to repair synthesis (Fig. 3D). RPA_RPA2D is therefore functionally active in supporting DNA replication in vitro. RPA_RPA2D was also found to bind normally to short ssDNA oligonucleotides (4). These results are not completely unexpected, as it was shown previously that the RPA phosphorylation state does not appreciably affect the ability of RPA to function in viral DNA replication or in DNA repair (2, 7, 36). In sum, mutation of the seven serines and one threonine in the N terminus of RPA2 to negatively charged aspartate residues does not have any apparent effect on the inherent activity of the heterotrimeric protein.

We next examined the possibility that expression of the RPA2D mutant generates a signal that shuts down cellular DNA synthesis and thus indirectly prevents RPA2D from associating with chromatin. To address this issue, cells were transfected with the RPA2wt or RPA2D expression construct...
FIG. 3. RPA2D-containing RPA heterotrimers are replication competent. (A) U2-OS cells were transfected with empty vector (lanes 1 and 4), myc-RPA2wt (lanes 2 and 5), or myc-RPA2D (lanes 3 and 6). Lysates were prepared from each batch of transfected cells, and the lysates were subjected to immunoprecipitation using anti-RPA1 antibodies. Immunoprecipitates (IP) (lanes 1 to 3) and aliquots of the lysates (lanes 4 to 6) were then analyzed for the presence of RPA2 by Western blotting analysis using RPA2 antibodies (which recognize both transfected and endogenous RPA2). (B and C) RPA heterotrimers that contained either RPA2wt (lane 1) or RPA2D (lane 2) were expressed in E. coli, purified, and analyzed by SDS-PAGE and Coomassie blue staining (B). The purified RPA was then assayed for the ability to support SV40 DNA replication in combination with an AS65 fraction purified from HeLa cells (51) (C). The open triangle shows that only background levels of DNA synthesis occur in reactions containing RPA2wt but lacking T antigen. Similar results were observed using RPA2D (D) SV40 DNA replication reactions were performed in the presence of [α-32P]dCTP to label the replication products as described in Materials and Methods. The reaction mixtures contained 600 ng of either RPA-RPA2wt (lanes 1, 2, 4, and 5) or RPA-RPA2D (lanes 3 and 6) and either lacked T antigen (lanes 1 and 4) or contained 750 ng of T antigen (lanes 2, 3, 5, and 6). After isolation, the DNA replication products were first linearized by restriction digestion and then either mock treated (lanes 1 to 3) or incubated with DpnI to cleave nonreplicated DNA (lanes 4 to 6). The digestion products were then subjected to agarose gel electrophoresis, and the images of the ethidium bromide (EtBr)-stained gel (to show the total level of DNA) and the autoradiograph of the gel (to visualize 32P-labeled reaction products) are provided. The observed bands correspond to the linearized SV40 origin-containing plasmid.

and pulse-labeled with BrdU. The cells were then subjected to FACS based on three signals: the level of myc-RPA2, DNA content, and BrdU incorporation. In addition to confirming that the two RPA2 variants were expressed at comparable levels (Fig. 4A and C), it was found that the percentages of cells in S phase were similar regardless of whether the cells were transfected with RPA2wt (Fig. 4B), RPA2D (Fig. 4D), or empty vector (not shown). Although the percentage of cells in S phase was somewhat high compared to other experiments, perhaps because of transfection conditions, the fractions of cells in S phase were routinely found to be similar for RPA2wt and RPA2D. We conclude that expression of RPA2D does not significantly affect cell cycle progression.

RPA2D is unable to complement the loss of endogenous RPA2. The data presented above suggested that the RPA2wt subunit, but not RPA2D, would be able to complement the loss of endogenous RPA2 and support chromosomal DNA replication. To test this possibility, cells were depleted of cellular RPA2 by using an siRNA molecule directed against the 3′ noncoding sequence of RPA2. The RPA2 expression cassettes do not contain the siRNA-targeted sequences, and hence the myc-RPA2 RNA produced from these vehicles is resistant to siRNA-mediated degradation. Visualization of RPA2 in these siRNA-treated cells by epifluorescence microscopy showed an apparent reduction of the RPA2 signal to nearly background level in >90% of the cells (compare Fig. 5D with A). Western blotting analysis indicated that RPA2 levels were reduced by >95% (data not shown). Upon cotransfection with myc-RPA2wt, a significant fraction of the cells demonstrated a robust myc signal bound to chromatin, with the pattern of binding similar to that seen in replicating cells (Fig. 5C). In contrast, little or no myc-RPA2D was found associated with chromatin (Fig. 5F), even though comparable levels of RPA2wt and RPA2D expression were detected in nonextracted cells (Fig. 5B and E, respectively). We therefore conclude that RPA2D, unlike RPA2wt, is unable to complement the loss of endogenous RPA2 and support DNA replication. These data also indicate that RPA2D is not prevented from binding ssDNA because of competition with the endogenous RPA but rather is inherently unable to productively interact with the DNA replication machinery.

RPA association with replication centers is dependent on the RPA2 N terminus negative charge. We next examined whether mutation of particular serine or threonine residues to aspartate was responsible for the lack of RPA2D association with replication centers or whether it was a consequence of the heightened negative charge at the RPA2 N terminus. We first constructed serine-to-aspartate substitutions at the cyclin-ckd2 sites S23 and S29 (RPA2D2) (Fig. 6G). S29 is invariably modified in each form of phosphorylated RPA (53), and thus, the RPA2D2 mutant resembles the form found in the initial steps of the RPA phosphorylation pathway. Further intermediate RPA2 mutants were designed to roughly follow the phosphorylation pathway, as suggested by the data of Zernik-Kobak and colleagues (53). However, it must be mentioned that the exact pathway of RPA2 modification from the hypophosphorylated to the hyperphosphorylated form is not known, and it is unlikely that a strict order of modification occurs in vivo. Additional serine-to-aspartate changes were generated in the background of the RPA2D2 mutant, with a total of three (RPA2D3...
and RPA2D31), four (RPA2D4 and RPA2D41), or five (RPA2D5) positions mutated. The sites mutated in these RPA2 variants are also found to be modified in RPA with an intermediate phosphorylation state in vivo.

Transfection of U2-OS cells indicated that all of the intermediate RPA2 mutants were expressed at similar levels (Fig. 6A and B and data not shown). Relative to RPA2wt, the RPA2 mutants with two or three Ser→Asp changes had two notable effects: (i) a modestly reduced fraction of transfected cells showing mutant RPA2 bound to chromatin (Fig. 6H) and (ii) a reduction in the intensity of RPA2 bound to chromatin (see below). More dramatic effects were observed when four or five serines were converted. For RPA2D41, the fraction of cells with significant chromatin binding was threefold less than for RPA2wt, and this fraction was reduced to 8% for the RPA2D5 mutant (Fig. 6H). The intensities of chromatin staining for the intermediate RPA2 mutants were also greatly reduced in individual cells, as demonstrated by comparing the average staining patterns of cells transfected with RPA2wt and RPA2D41 (Fig. 6C and D, respectively [taken with identical exposure times]).

The decrease in association of RPA2 with replication centers was most strongly correlated with the number of aspartate residues rather than with changes at any particular positions. The notion that the mutation of serines to aspartates per se (i.e., irrespective of the changes in the RPA2 negative charge) causes decreased RPA binding to replication centers is argued against because the N terminus of RPA2 is not critical for DNA replication in vitro for mammalian RPA (23) or in vivo for yeast RPA (38). These data therefore suggest that the increase in net negative charge afforded by the increased number of aspartate residues is the primary factor regulating RPA binding to chromatin. Although the location of the aspartate residues did not appear to have major effects on RPA2 activity, we did note that mutation of the S33 site, known as a consensus sequence for PIKKs, appeared to have a somewhat more deleterious effect.

RPA2D is recruited to DNA damage foci following genotoxic stress. Under DNA damage conditions, a significant change occurs in the nuclear distribution of RPA, with the more diffuse punctate pattern seen during DNA replication transform-
ing to bright, well-distinguished foci. In this state, RPA colocalizes with a number of repair and checkpoint proteins (e.g., ATR and Rad51) and is thought to demarcate the sites of DNA repair and/or unrepairable lesions (19, 20, 39, 54). Such stress conditions cause a subset of the endogenous RPA pool to become hyperphosphorylated (see below). We therefore reexamined the behavior of RPA2_D and RPA2_A in cells undergoing genotoxic stress.

Cells were transfected with the RPA2wt, RPA2_A, or RPA2_D expression construct and then treated with CPT. CPT inhibits topoisomerase I, indirectly causing DNA double-strand breaks, and leads to rapid and massive RPA phosphorylation (42). Similar to RPA2wt (Fig. 7A to C), the RPA2_A variant colocalized with RPA1 in bright foci following CPT treatment (Fig. 7G to I). Very similar foci were observed for endogenous RPA2 (not shown). Thus, the phosphorylation-defective RPA2_A variant is apparently competent to bind chromatin both in normal (above [Fig. 2L]) and in stressed cells.

In sharp contrast to the inability of RPA2_D to stably associate with replication centers, CPT treatment caused the RPA2_D variant to colocalize with RPA1 in DNA damage foci (Fig. 7D to F). The number and distribution of these foci, as well as the intensity of staining, were indistinguishable from those observed with the RPA2wt (and RPA2_A) construct. Thus, although the RPA2_D mutant is unable to localize to replication centers, this defect does not extend to the involvement of RPA2_D in the DNA damage response.

We determined if the CPT-dependent recruitment of RPA2_D to DNA damage foci was applicable to other stresses. We tested HU and aphidicolin, agents that do not directly cause DNA damage but rather result in stalling of the DNA replication fork. As cells were incubated with HU from 1 to 3 h (Fig. 8E and F), a progressive increase in RPA2_D association with chromatin was observed, with most cells demonstrating a dispersed staining pattern. A fraction of cells exhibited distinctive foci, and these showed significant colocalization with γ-H2AX, the phosphorylated form of histone variant H2AX that is a marker for sites of DNA damage (Fig. 8I to L) (40). Similar effects of HU were noted on cells transfected with RPA2wt (Fig. 8A and B). In contrast, treatment with aphidicolin for 3 h did not stimulate RPA2_D association with chromatin (Fig. 8M; data not shown), demonstrating reduced toxicity of aphidicolin relative to HU under these conditions. Exposure to ionizing radiation (10 Gy) gave rise to staining patterns of RPA2wt and RPA2_D similar to that found with CPT (data not shown).

In the functional absence of the budding yeast homologs of ATR and its downstream effector Chk1 (Mec1p and Rad53, respectively), replication forks have a greater propensity to collapse when encountering DNA damage, yielding unregulated production of long ssDNA regions (32, 43, 45). We therefore hypothesized that addition of caffeine, an inhibitor of the ATR-ATM-dependent checkpoint response (21, 41), to HU-treated cells would similarly lead to replication fork degradation. This in turn would cause faster induction of DNA damage foci and of RPA2_D localization. To test this hypothesis, RPA2wt- or RPA2_D-transfected cells were treated with HU for 1 or 3 h in the presence of caffeine. Particularly for RPA2_D, addition of caffeine dramatically increased the number and intensity of RPA2 foci at both the 1- and 3-h time points (Fig. 8G and H). Quantification of the effects on myc-RPA2 localization demonstrated that caffeine greatly increased the frac-
FIG. 6. Increase in negative charge at the RPA2 N terminus decreases frequency of myc-positive cells. (A to F) Cells were transfected with myc-RPA2wt or myc-RPA2D41 as indicated. Representative epi-fluorescence images showing total myc-RPA2 staining (no extraction) (A and B), chromatin-bound myc-RPA2 (C and D), and chromatin-bound RPA1 (E and F) are shown. (G and H) Serines and threonine (boldface and underlined) at the potential phosphorylation sites in the N terminus of the RPA2 subunit were replaced with alanines (A) or aspartates (D) as indicated. Cells expressing these myc-tagged RPA2 mutants were then analyzed by immunofluorescence microscopy for the presence of myc-RPA2 bound to chromatin and in parallel reactions, for transfection efficiency. The relative frequencies of myc-RPA2 positive cells were calculated as described in Materials and Methods.
tion of HU-treated cells with significant RPA2D and RPA2wt signals (Fig. 8M).

The effects of these various stress conditions on endogenous RPA phosphorylation were also examined (Fig. 8N). Those stress conditions that resulted in significant RPA2D chromatin binding also caused increased phosphorylation of endogenous RPA2, although CPT caused modification of a greater fraction of the RPA pool, as well as phosphorylation of more RPA2 sites, than HU. Enhanced phosphorylation of RPA following a 1-h treatment with HU and caffeine was occasionally seen.

Consistent with our results showing the inability of aphidicolin to stimulate the chromatin binding of RPA2D, aphidicolin also did not induce RPA2 phosphorylation. Because caffeine has been demonstrated to be an inhibitor of ATM and ATR kinase activities (21, 41), the observed hyperphosphorylation of RPA probably results from the caffeine-insensitive activity of DNA-PK that is stimulated by collapsed replication forks. However, we note that a recent study found that caffeine can inhibit the checkpoint response without inhibiting ATR-ATM kinase activity in vivo (11), leaving open the possibility that these kinases may still be responsible. In any case, these data indicate that the rate and extent of RPA2D (and RPA2wt) localization to sites of DNA damage correlate with the degree of DNA damage sustained during stress.

**Localization of endogenous hyperphosphorylated RPA.** The properties of endogenous hyperphosphorylated RPA were examined using an antibody generated against an RPA2 peptide doubly phosphorylated on serine residues 4 and 8. Lysates prepared from untreated or CPT-treated U2-OS cells were probed with either a general RPA2 antibody or the pSer4/pSer8-RPA antibody (Fig. 9J). The phosphospecific antibody selectively recognized a species from CPT-treated cells that comigrated with hyperphosphorylated RPA2 by Western blotting analysis. Prior incubation of the CPT-treated lysates with phosphatase resulted in the loss of both the hyperphosphorylated RPA2 form and reactivity by the phosphospecific antibody. We conclude that the phosphospecific antibody recognizes a hyperphosphorylated RPA2 species that is modified on Ser4 and Ser8.

The phosphospecific antibodies were used to examine the localization of the pSer4/pSer8 form of RPA in untreated U2-OS cells and in cells treated with HU alone or with HU and caffeine. In control cells or in cells treated only with HU (Fig. 9D and E), little if any pSer4/pSer8-RPA staining was detected. Following
FIG. 8. Collapse of DNA replication forks stimulates RPA loading to damaged DNA. (A to H) U2-OS cells were transfected with plasmids expressing either myc-RPA2wt or myc-RPA2Das indicated for 48 h. The cells were then treated with 2.5 mM HU for either 1 (A and E) or 3 (B and F) h in the absence of caffeine or treated with HU for 1 (C and G) or 3 (D and H) h in the presence of 5 mM caffeine. The cells were Triton X-100 extracted and fixed and then stained for myc-RPA. (I to L) Colocalization of myc-RPA2D with sites of DNA damage. U2-OS cells were transfected with a myc-RPA2D-expressing plasmid. Forty-eight hours posttransfection, the cells were treated with 2.5 mM HU for 3 h and then extracted with 0.5% Triton X-100 and fixed. The cells were stained with BrdU and H2AX (I) and myc-RPA (J). The staining pattern of a representative cell and the image of the merged staining patterns (K) are provided. (L) One section (boxed) of the composite image is shown enlarged to reveal the degree of signal overlap. (M) Graph showing the fraction of myc-RPA2-transfected cells with detectable RPA signal. The fractions of cells showing chromatin-bound RPA were quantified by visual inspection of 100 to 200 cells. The bar graph values were calculated as described in Materials and Methods. Note that although RPA2wt staining is consistently detected in a greater fraction of cells than RPA2Das staining, this result is expected because RPA2wt can be observed both at replication centers and at DNA damage foci while RPA2Das localizes only to damage foci. (N) Effects of stress and caffeine treatment on endogenous RPA2 phosphorylation. U2-OS cells were mock treated (Mock) or treated with either 0.5 mM CPT for 1 or 3 h, 2.5 mM HU for 1 or 3 h, 5 mM caffeine (C) for 3 h, 2.5 mM HU for 1 or 3 h in the presence of 5 mM caffeine, or with 0.5 mM aphidicolin (A) for 3 h and 0.5 mM aphidicolin for 1 or 3 h in the presence of 5 mM caffeine. The cell cycle was arrested with 2.5 mM HU for either 0.5 h (C and G) or 3 h (D and H) in the absence of caffeine or treated with HU for 1 h (E and F) in the presence of caffeine. The cells were then extracted with NP-40 buffer, and the cell lysates were analyzed by Western blotting with antibodies directed against RPA2. The positions of nonphosphorylated RPA2 and phosphorylated RPA2 (P-RPA2) are indicated by arrows. The results are representative of three independent experiments.
treatment with both HU and caffeine, the cells showed a dramatic increase in pSer4/pSer8-RPA staining (Fig. 9F). The staining pattern was nearly identical to that of total RPA2 (compare Fig. 9C and F), and also showed good overlap with γ-H2AX staining (data not shown). The colocalization of pSer4/pSer8-RPA with sites of DNA synthesis was also examined. Cells were treated with CPT and then incubated with BrdU. The areas of pSer4/pSer8-RPA staining did not colocalize with sites of remaining DNA synthesis.
synthesis to any significant degree (Fig. 9L). A majority of the RPA pool is hyperphosphorylated under these conditions (Fig. 8N), rendering similar experiments using general RPA2 antibodies uninformative. We conclude that the hyperphosphorylated form of RPA localizes only to chromatin following DNA damage and is not significantly associated with sites of chromosomal DNA synthesis.

**DISCUSSION**

We find that the RPA2D mutant that mimics the hyperphosphorylated state is prevented from stable association with replication centers in vivo. The lack of association with sites of DNA synthesis is also observed for endogenous hyperphosphorylated RPA and is not a result of competition with the nonphosphorylated protein. Importantly, the RPA_{RPA2D} protein has activity equivalent to the wild-type protein both in ssDNA binding (4) and in SV40 DNA replication in vitro. The inherent activity of hyperphosphorylated RPA or RPA_{RPA2D} in vivo also appears normal because genotoxic stress causes these RPA species to localize to DNA damage foci similarly to endogenous RPA2 and RPA2\_wt. Our data therefore indicate that the chromosomal DNA replication machine has the ability to discriminate between RPA species with different phosphorylation states. In addition to providing a means to regulate RPA loading and hence DNA replication, RPA phosphorylation also has the potential to mark sites of DNA damage or replication stress for recruitment of repair factors.

Our data suggest a novel feature of eukaryotic DNA replication, namely, that RPA is actively loaded onto the ssDNA by the chromosomal replication machinery. This model arises from the fact that RPA_{RPA2D} and by inference hyperphosphorylated RPA, is inherently active in binding the ssDNA at a DNA replication fork but is unable to do so in vivo. The most logical explanation is that, as the duplex DNA is unwound by the advancing DNA helicase, the hypophosphorylated RPA is loaded onto the ssDNA by protein components of the replication fork machinery. One could easily envision, for example, that the minichromosome maintenance (MCM) complex, suggested to be the eukaryotic replicative helicase (29) and known to interact with RPA (55), would load RPA molecules in a step-by-step fashion as the ssDNA is generated. Selective binding of nonphosphorylated RPA (i.e., endogenous RPA, RPA_{RPA2D} or RPA_{RPA2D}) to MCM would therefore allow this RPA species to bind only to unwound DNA. (The MCM complex is not involved in SV40 DNA replication.) However, RPA interacts with various proteins, including the DNA polymerase α-DNA primase complex (14), and RPA phosphorylation has been found to inhibit the association with DNA polymerase α (34). Thus, discrimination of the RPA phosphorylation state can be achieved by these or other replication factors. One alternative model that does not require concerted RPA loading would involve a discrimination filter that prevents access of the phosphorylated RPA to the replication fork. The nature of such a filter would be difficult to envisage.

DNA-damaging stress relieves the inhibition of RPA_{RPA2D} chromatin binding and causes RPA_{RPA2D} association with DNA damage foci, as evident by colocalization with γ-H2AX. That HU causes RPA_{RPA2D} foci to form and increases the level of RPA2 phosphorylation while aphidicolin does neither indicates that replication fork blockage is not sufficient for RPA_{RPA2D} chromatin binding but that the presence of DNA damage or aberrant replication fork structures is also required. This conclusion is strengthened by our observation that inhibition of ATR- or ATM-mediated checkpoint response by caffeine stimulates the rate of RPA association with DNA damage foci. Mutation of MEC1, the *Saccharomyces cerevisiae* ATR homolog, is known to cause the collapse of DNA replication forks that have been stalled by treatment with HU or methyl methanesulfonate (32, 45), and such treatment leads to the production of long ssDNA regions (43). Because of the high affinity of RPA for ssDNA (27, 52), we propose that the increased availability of ssDNA releases the constraints on RPA loading seen during normal S-phase progression. Thus, under damage conditions, the RPA phosphorylation state no longer regulates the association of RPA with chromatin.

Our data indicate that hyperphosphorylated RPA is preferentially associated with sites of DNA damage. The specific association of repair factors with this modified form of RPA would therefore provide a mechanism to recruit repair factors to sites of DNA damage. Interestingly, the ATRIP-ATR complex has been found to sense damaged DNA by recognition of RPA-ssDNA complexes. Clearly, RPA phosphorylation has the potential to regulate the binding of ATRIP-ATR and thereby modify the cellular checkpoint response. Although our examination of RPA2\_α expression did not detect any notable effects on cell cycle progression, it will be interesting to examine whether RPA2\_α and RPA2\_α expression in cells lacking endogenous RPA alters cellular proliferative capacity or response to DNA damage.

Finally, our data indicate that hyperphosphorylation of RPA can limit its ability to support chromosomal DNA replication. It is unlikely that this mechanism alone could cause significant reductions in the level of DNA synthesis during genotoxic stress. Under severe stress conditions, such as 1-h exposure to 1 μM CPT (Fig. 8N) or irradiation with 30 J of UV light/m2 (53), the hyperphosphorylated form of RPA contributes ~50% of the total RPA pool prepared from asynchronous cells. Even though the fraction of hyperphosphorylated RPA may be higher in S-phase cells, these data suggest that enough hypophosphorylated RPA would be available to sustain DNA replication. That being said, we and others have found that stress conditions also lead to the inhibition of RPA activity by other processes (9, 30, 48), including sequestration of RPA by nucleolin (12, 47). Combined, these data suggest that inhibition of RPA activity by multiple mechanisms can serve to repress chromosomal DNA replication following stress.

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Views and Commentaries

The Toposome
A New Twist on Topoisomerase IIα

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As a small boy, I frequently visited nearby woods where a favored pastime was to overturn rocks on the forest floor. More often than not, I would discover a new sub-rocca world teeming with life. Nowadays, having less time to gambol through the woods and my curiosity focusing on biological questions, a similar enjoyment can be obtained from leering of trees of known factors to unanticipated processes. An example is provided by various recent findings relating to Topoisomerase IIα (Topo II), including the work of Lee and colleagues who found that this enzyme can be isolated in a complex containing factors involved in RNA processing and transcription.1

Topo II has been known to play key roles at two primary stages during the cell cycle. In preparation for mitosis and cytokinesis, Topo II acts in concert with the condensin complex2 to condense the chromosomes 10,000-fold into the mitotic structures. A second requirement for Topo II arises because the process of DNA replication invariably leads to entanglement of the chromosomes, links between DNA molecules that must be resolved or the genomic information becomes endangered. Decatenation activity is primarily provided by Topo II which functions as the sister chromatids are preparing to segregate to the opposite cellular poles. Both condensation and decatenation utilizes the ATP dependent strand passage activity of Topo II.

When biochemically screening for novel nuclear kinases that modify RNA processing factors containing serine/arginine (SR) dipeptide repeats, Lee et al isolated a large complex, termed the toposome, containing a known SR kinase, SRPK.1 Modification of SR proteins by members of the SRPK and functionally related Chk/Sty families promotes nuclear import and recruitment to nascent transcripts for RNA splicing.3 In addition to containing other factors with known roles in transcription (RNA helicase A and SsRP31, pre-mRNA splicing (PRP8 and hU1RNP C), and RNA processing (RHI/Gu), the toposome complex also contained Topo II. That the association is functionally meaningful is indicated by the finding that the toposome has higher decatenation activity on chromatin substrates compared to isolated Topo II, and forms in a cell cycle-regulated manner with highest levels detected during mitosis.

The findings have a touch of déjà vu to the functional association of Topo II with condensin, a pentameric complex comprised by SMC2, SMC4, CapG, CapD2, and CapH/Barren. The CapH/Barren subunit of condensin can both bind Topo II and stimulate its relaxation activity, and these two factors colocalize on mitotic figures.4 Conditional knockouts of the SMC2 subunit in chicken cells are defective in proper localization of Topo II on mitotic chromosomes, and reduce Topo II levels at centromeres.5 Future characterization of the toposome will require similar investigation of the possible localization of Topo II with the other toposome factors on mitotic chromosomes. Such studies will not only reveal whether the toposome is bound to chromatin during mitosis, but indicate if the toposome complex occurs at all Topo II bound sites or only a subset of these targets. It is known that the sequences with which Topo II associates in vitro are somewhat different than those it binds in vivo. Moreover, chromatin structure has a strong influence on the binding preference of Topo II to centromeric sites.6 Thus, it is conceivable that formation of the toposome alters the binding preferences of Topo II to particular chromatin structures. If apparent toposome complexes exist on mitotic chromosomes, the effect of disruption or inhibition of toposome components other than Topo II should provide unambiguous answers as to whether the major role of the toposome relates to the known roles of Topo II in chromosome condensation and separation.

As mentioned above, Lee et al observed that the toposome apparently assembles as cells are entering mitosis, as low levels of the complex were seen in cells at the G1/S boundary or in S phase. Topo II has heightened phosphorylation at the G1/M transition and this is apparently catalyzed by protein kinase C (PKC) and p34cdc2.7,8 Therefore, it would not be unexpected that modification increases the affinity of Topo II for other toposome components.
Test of the effects of mutation of the PKC and p34cdc2 sites on Topo II would clarify the significance of phosphorylation on toposome formation, and separate these effects from direct changes in Topo II activity per se. Recent evidence also demonstrates that S. cerevisiae and Xenopus Topo II undergo conjugation to SUMO, with disruption of this modification resulting in defective sister chromatid dissociation and centromere cohesion.9,10 In Xenopus, SUMO conjugation occurs during mitosis,10 and this modification clearly has the potential to regulate Topo II-protein interactions. Other toposome components are also modified by phosphorylation, acetylation, etc., and reciprocal changes in the modification patterns of these other factors could also regulate toposome formation.

What is the significance of the association of the RNA splicing and processing components with the toposome? Recent data by other laboratories lead to the suggestion that the toposome may be involved in heterochromatin formation. Topo II has a dynamic association with nuclear speckles that are thought to represent deposits of splicing factors, and this pool is in equilibrium with Topo II involved in RNA polymerase II transcription.11 Topo II also localizes to heterochromatic regions in a DNA replication-dependent fashion.11 These data can be coupled to the finding that two different histone deacetylases (HDAC1 and HDAC2) physically interact with Topo II with reciprocal regulation of their activities.17 Because of the emerging role for short noncoding RNA molecules in assembling heterochromatin,13 a possibility to be entertained is that Topo II is coupled to the RNAi machinery via toposome components. The toposome would therefore facilitate the maintenance of heterochromatin structure following passage of the DNA replication fork.

Lastly, because Topo II is an abundant factor on chromatin during mitosis, association with Topo II in the toposome may allow for the proper distribution of splicing factors to daughter cells as passenger proteins. During mitosis, a large number of factors, many involved in RNA processing or ribosome biogenesis such as fibrillarin, nucleophosmin, and ribosomal protein S1, become associated with condensed chromosomes.14 Among other roles (e.g., protection of mitotic chromosomes), chromosome association has been postulated to allow relatively equal partitioning of such factors to daughter cells.

Clearly, many questions are raised by the intriguing association of Topo II with RNA processing and transcription factors in the toposome. The literature is now becoming ripe with examples of proteins that have distinct activities in, at first glance, unrelated processes. The work of my own laboratory finds that nucleolin, a protein with established roles in ribosome biogenesis and pre-RNA processing, can also inhibit DNA replication following heat shock and genotoxic stress.15,16 As our understanding of biological processes matures, an important but arduous goal will be to decipher how the disparate processes of the cell are integrated into a unified whole.

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