Nucleolar Targeting of RelA(p65) Is Regulated by COMMD1-Dependent Ubiquitination

Hazel C. Thoms, Carolyn J. Loveridge, James Simpson, Alexandra Clipson, Karina Reinhardt, Malcolm G. Dunlop, and Lesley A. Stark

Abstract

Stimulation of the NF-κB pathway can have proapoptotic or antiapoptotic consequences, and one mechanism that determines the outcome is the nuclear distribution of RelA. Certain stress stimuli induce nucleolar accumulation of RelA thereby mediating apoptosis, whereas others induce nucleoplasmic accumulation and inhibition of apoptosis. Here we investigated the mechanisms that regulate the nuclear distribution of RelA, specifically, the role of the ubiquitin/proteasome system. We found that stress-induced nucleolar translocation of RelA is preceded by ubiquitination of the protein. We also found that chemical proteasome inhibitors induce the ubiquitination and nucleolar translocation of RelA and that this is required for the apoptotic response to these agents. We show that the RelA nucleolar localization signal (amino acids 27–30) is a critical domain for ubiquitination of the protein but that the lysine residue within this motif is not a direct target. We show that RelA binds COMMD1, the rate-limiting component of the RelA ubiquitin ligase complex, in response to stress. Furthermore, we show that overexpression of COMMD1 promotes stress-mediated nucleolar targeting of RelA, whereas knockdown of COMMD1 blocks this effect, causing RelA to remain in the nucleoplasm. These data identify a new role for COMMD1 in regulating the nuclear/nucleolar distribution of RelA and suggest that ubiquitination acts as a signal for transport of RelA to the nucleolus. These findings have relevance to the design of chemopreventive/anticancer agents that act by targeting RelA to the nucleolar compartment. Cancer Res; 70(1); 139–49. ©2010 AACR.

Introduction

The NF-κB transcription factor generally exists as a heterodimer of the p50 and RelA(p65) polypeptides bound in the cytoplasm by the inhibitor protein IκB(1, 2). Following cellular stimulation, IκB is phosphorylated and then degraded by the proteasome, allowing NF-κB to translocate to the nucleus and regulate the expression of downstream genes (3). A central function of NF-κB, in particular the RelA sub-component, is the regulation of cellular growth and apoptosis (4, 5). However, this is complex because stimulation of NF-κB can have both proapoptotic and antiapoptotic effects, dependent on the context. For instance, persistent activation of NF-κB is a recognized contributory factor in a number of common cancers, whereas stimulation of the pathway by agents such as UV radiation, serum withdrawal, and cell stress promotes cell death (6, 7).

Despite the importance of NF-κB in regulating cellular growth, proliferation, and survival, the ability of the NF-κB pathway to have both proapoptotic and antiapoptotic consequences makes it an attractive therapeutic target. One potential strategy is to induce the cytoplasmic release of NF-κB or to activate the degradation of NF-κB proteins to achieve an anti-apoptotic function (8–10). One pathway by which this can be done is via the ubiquitin-proteasome system (11). The mechanism by which NF-κB is degraded varies depending on the context, and appear to be determined by the signals that trigger the induction of apoptosis (11). NF-κB transcriptional activity and apoptosis is the resultant of both transcriptional activity and transcriptional activity and mRNA translation (12). One post-induction mechanism that we have identified for regulating NF-κB transcriptional activity and apoptosis is the nuclear/nucleolar distribution of RelA. We have shown that in response to specific stress-inducing stimuli, including UV-C radiation, serum deprivation, and aspirin and related agents, RelA translocates from the cytoplasm to the nucleoplasm and then to the nucleolus (11–13). We identified a signal at the NH2 terminus of RelA that is required for nucleolar translocation of the protein and showed, using a dominant-negative mutant deleted for this domain, that nucleolar translocation of RelA is causally involved in the down-regulation of NF-κB–driven transcription and the induction of apoptosis (11). Understanding the pathways that regulate nucleolar distribution could inform on the design of molecules that modulate these pathways and, thus, NF-κB transcriptional activity and apoptosis. Although nucleolar localization signals are thought to be involved, the signals that regulate the nucleolar localization of proteins have yet to be fully defined (14–16). One pathway that has been implicated is the ubiquitin-proteasome system.
Proteins such as c-myc, p53, and ADP ribosylation factor translocate to the nucleus in response to proteasome inhibition, leading to the suggestion that nuclear compartmentalization regulates protein stability (17–20). This is of particular interest because RelA undergoes ubiquitination and proteasome-mediated degradation in the nucleus (21–26). Therefore, we set out to determine the role of the ubiquitin-proteasome system in the nuclear/nucleolar distribution of RelA and apoptosis.

Materials and Methods

Cell culture, reagents, and fractionation. SW480 colon cancer cell culture, HeLa cervical cancer cell culture, and treatment with aspirin have been described (11, 27). SW480 cells that stably express GFP-RelA WT or Δ27–30 were generated by selection in G418. The proteasome inhibitors MG132 and lactacystin (Calbiochem), TNF and TNF-related apoptosis-inducing ligand (TRAIL; R&D Systems), and calyculin A (Cell Signaling Technology) were prepared as per manufacturers' instructions. Cell fractions were isolated from SW480 cells as described previously (11, 13).

Proteasome and reporter assays. Proteasome activity was measured in lysates of cellular fractions (25 μg) using a 20S Proteasome Fluorometric (AMC) Assay Kit as per manufacturer's instructions (Chemicon). For each sample, standards provided were used to convert relative fluorescent units to micromolar concentrations of free AMC, and then results were presented as percentage of basal (nontreated) proteasome activity. All results shown are the mean of duplicates of at least three independent experiments ± SE.

Western blot analysis. Extracts (30 μg) were resolved on 5% to 10% SDS polyacrylamide gels, and immunoblots done following standard procedures. The following primary antibodies were used: p65(C-20), p65(F-6), nucleolin (C23), fibrillarin, glutathione S-transferase (GST), and green fluorescent protein (GFP; all from Santa Cruz); His [generated in-house (purified form)]; ubiquitin (rabbit antibody; Dako Cytomation); ubiquitin (mouse monoclonal; Stressgen); filamin A (Bethyl Laboratories); phospho-p65 (Ser468) and phospho-p65 (Ser536) (Cell Signaling Technology).

Plasmids. The wild-type (WT) and R7 ΔHis-ubiquitin (Ub) plasmids, HA-Ub, and the 3enhancer CONA (3xUb ConA-Luc) reporter construct were supplied by Prof. R. Hay (28, 29). The pCMV-β-galactosidase plasmid is commercially available (Promega). The GFP expression vectors used have been described (11). The GST-COMMD1 expression vector and corresponding control were gifts from Dr. E. Burstein and have been described (30).

Transfections. Plasmid transfections were carried out using Lipofectin (Life Technologies, Inc.; ref. 11). After 24 to 48 h of recovery, cells were treated with aspirin or proteasome inhibitors as specified in the text.

For transfection of small interfering RNA (siRNA), cells were transfected with control (scrambled) or COMMD1 [#1, AAGTCATATTGCGTCTGCAAGC(30); #2, GACAGGAATCTGAAATTTCTGT] siRNA using Lipofectamine 2000 (Life Technolo-

gies) as per manufacturer's instructions. Cells were recovered for 48 h before treatment with aspirin.

Immunoprecipitation. Immunoprecipitation assays were done using 500 μg of whole-cell lysate as previously described (31). Rabbit polyclonal anti-RelA (Santa Cruz) and anti-ubiquitin (Stressgen) were used to immunoprecipitate the appropriate protein. Mouse or rabbit IgG (preimmune serum) was used as control. Complexes were resolved by SDS-PAGE and then analyzed by Western blot analysis. Precipitation of ΔHis-tagged Ub using Ni-agarose (29) and GST-COMMD1 using glutathione-Sepharose (30) was carried out as previously described. Ubiquitinated and COMMD1-bound RelA were detected using anti-RelA Western blot analysis. Anti-histidine and GST were used as controls.

Live-cell imaging. The localization of GFP-tagged proteins was analyzed in snap shots of live adherent cells using an Axiovert 100 inverted microscope (Zeiss) at an excitation wavelength of 488 nm. Images were captured and processed using IPLab 3.6 with scripts written in-house.

Immunocytochemical staining. Immunocytochemistry was done using antibodies against RelA, nucleolin, and fibrillarin as described (11). Fluorescent microscopy was done with a Zeiss Axioplan microscope, 63× Plan Neofluor objective, and Chroma 83000 filter set. Images were captured using IPLab Spectrum 3.6. The percentage of cells showing nuclear RelA was determined in at least 200 cells from at least five fields of view in three independent experiments or as specified.

Apoptosis assays. Apoptosis assays were carried out on cells transfected with GFP-tagged constructs as described (11). At least 250 transfected cells (detected in FITC channel) were examined for Annexin V binding (detected in Texas red channel) for each sample. All results presented are the mean of at least three experiments ± SE.

Results

Nuclear accumulation of RelA and proteasome activity. We hypothesized that nuclear accumulation of RelA may be caused by inhibition of proteasome activity in the nucleolus itself (17). Therefore, using aspirin as a model stress-inducing stimulus of the NF-κB pathway, we investigated the association between nuclear accumulation of RelA and proteasome activity. We found that, as previously described (32), aspirin mediated a partial reduction in total cellular proteasome activity (Fig. 1I). This reduction was biphasic and was initially observed within 30 minutes of treatment, which preceded an accumulation of ubiquitinated proteins within the cell (Fig. 1B and C). Consistent with our finding that aspirin stimulates the NF-κB pathway, Western blot analysis indicated that partially inhibiting proteasome activity (using low-dose proteasome inhibitor MG132) was insufficient to block signal (TNF)-mediated degradation of IκBα (data not shown).

Next, we measured proteasome activity in the nuclear (which, given the insolubility of nucleoli, predominantly represents nucleoplasmic proteins) and nucleolar fractions. The purity of isolated nucleoli was confirmed by light microscopy (data not shown) and by Western blot analysis,
which showed an enrichment for C23 and fibrillarin (nucleolar markers) and reduced lamin B (a nonnucleolar protein) in the nucleolar fraction (Fig. 1D). We found that basal levels of proteasome activity were low in nucleoli and increased in response to aspirin, whereas a decrease was observed in nuclei (Fig. 1D). These data indicate that RelA accumulation in the nucleolus is not a consequence of inhibition of proteasome activity in this compartment. However, decreased proteasome activity in the nucleoplasm may be a factor.

**Nucleolar translocation of RelA is preceded by ubiquitination of the protein.** We next determined whether the effects of aspirin on proteasome activity modulate ubiquitination of RelA. We found that aspirin induces a dose-dependent increase in high molecular weight forms of RelA, suggestive of the addition of multiple ubiquitin chains (Fig. 2A). Immunoprecipitation of endogenous RelA followed by anti-ubiquitin immunoblot confirmed increased amounts of ubiquitinated RelA in response to the agent (Fig. 2A). This was further confirmed using overexpression of 6His-tagged ubiquitin followed by precipitation of ubiquitinated proteins with Ni-agarose beads. Figure 2B shows that aspirin and MG132 (used as a positive control refs. 24, 25) mediate an increase in ubiquitinated forms of RelA in cells transfected with WT 6His-ubiquitin. This was not observed in cells transfected with R7-6His-ubiquitin (which is mutated at all lysine residues and thus cannot form polyubiquitin chains), indicating specificity (Fig. 2B). These data confirm that, like MG132, aspirin mediates the ubiquitination of RelA.

On examining the kinetic relationship between aspirin events, we found that ubiquitination of RelA coincided with nucleoplasmic accumulation of the protein, which precedes its appearance in nucleoli (Fig. 2C; ref. 11). Similar to aspirin, we observed an increase in high molecular weight forms of RelA in response to UV-C and found that this ubiquitination peaked when the protein was nucleoplasmic, prior to nucleolar translocation (Fig. 2D). In contrast, TNF, which induces RelA that remains in the nucleoplasm, had minimal effect on its ubiquitination status (Fig. 2D; ref. 11).
Nucleolar translocation of RelA in response to chemical inhibition of proteasome activity. The above data suggest that an accumulation of ubiquitinated forms of RelA in the nucleoplasm may act as a signal for nucleolar targeting of the protein. To further test this hypothesis, we examined the cellular localization of RelA in response to MG132. We found that RelA is targeted to nucleoli [as indicated by the nucleolar marker nucleolin (C23) and areas devoid of 4',6-diamidino-2-phenylindole (DAPI) staining] after treatment with this agent (Fig. 3A). Similar results were observed with the highly selective proteasome

Figure 2. Ubiquitination of RelA precedes nucleolar translocation of the protein. A, SW480 cells were treated with aspirin (0–5 mmol/L, 16 h). Top, the levels of high molecular weight [indicative of ubiquitination (Ub.)] and native RelA were determined by Western blot analysis of whole-cell lysates. Bottom, RelA was immunoprecipitated (IP) from lysates, and recovered protein analyzed using anti-ubiquitin Western blot analysis (WB Ub.). Immunoprecipitation with isotyped IgG controlled for specificity. Stripped gels were reprobed for RelA to monitor the amount of RelA immunoprecipitated (WB. RelA). B, SW480 cells were transfected with WT 6His-ubiquitin (ub) or the R7 mutant that cannot form polyubiquitin chains. Twenty-four hours after transfection, cells were either left untreated (NT) or treated with aspirin (5 mmol/L) or MG132 (25 μmol/L) for a further 16 h. His-tagged proteins were precipitated from whole-cell lysates using Ni-agarose beads and then subjected to anti-RelA and anti-histidine (his) Western blot analysis (WB). NS, nonspecific band. C, SW480 cells were treated with aspirin (10 mmol/L) for the times specified. Top, the levels of native and high molecular weight forms of RelA were determined using Western blot analysis. Bottom, the nuclear distribution of RelA was assessed by immunocytochemical staining (magnification, ×63). D, SW480 cells were exposed to UV-C radiation (40 J/m²) or TNF (10 ng/mL) for the times specified. Top, anti-RelA Western blot analysis done on whole-cell lysates. Bottom, immunomicrographs showing the cellular distribution of RelA (magnification, ×63).
ubiquitinated RelA in response to aspirin and MG132 in cells immunoprecipitation studies that indicated an increase in total protein ubiquitination, suggesting that this domain in the mutant protein did not inhibit aspirin/MG132 action. Using anti-RelA Western blot analysis, we found that the requirement for this domain in the ubiquitination of the protein. Data generated by immunocytochemical analysis of endogenous protein, whereas GFP-RelAΔ27–30 accumulates in aggregates in the nucleoplasm and is excluded from nucleoli (Fig. 4A). These data indicate the importance of this domain in stress- and proteasome inhibitor–mediated nucleolar translocation of RelA.

To further establish the relationship between ubiquitination and the nucleolar targeting of RelA, we next examined the requirement for this domain in the ubiquitination of the protein. Using anti-RelA Western blot analysis, we found that aspirin and MG132 induced an accumulation of high molecular weight forms of RelA in cells expressing GFP-RelAWT but induced no change in the ubiquitination status of RelA in cells expressing GFP-RelAΔ27–30 (Fig. 4B). Expression of the mutant protein did not inhibit aspirin/MG132–mediated increase in total protein ubiquitination, suggesting that this is a specific effect (Fig. 4B). These data were confirmed by immunoprecipitation studies that indicated an increase in ubiquitinated RelA in response to aspirin and MG132 in cells expressing GFP-RelAWT, which was abrogated in cells expressing GFP-RelAΔ27–30 (Fig. 4C). Expression of His-tagged ubiquitin in cells expressing GFP-RelA WT or Δ27–30, followed by Ni-agarose pull-down, further confirmed that aspirin induced an increase in ubiquitinated forms of GFP-RelAWT but had no effect on GFP-RelAΔ27–30.

We have previously shown that mutation of the single lysine residue within aa27–30 (K28R) has no effect on aspirin-mediated nucleolar translocation of the protein (11). We found that mutation of this lysine also had no effect on aspirin–proteasome inhibitor–mediated ubiquitination of the protein, suggesting that this is not a direct target (Fig. 4B and C). These data show that the same motif is required for ubiquitination and nucleolar translocation of RelA, providing further evidence that the two events are linked.

Nuclear translocation of RelA is required for proteasome inhibitor–mediated apoptosis. Previously, we reported that nuclear targeting of RelA regulates NF-κB–driven transcription and apoptosis (11). Consistent with these studies, NF-κB reporter assays confirmed the repression of NF-κB–driven transcription in response to proteasome inhibitors (Supplementary Fig. S1A), and showed that this repression occurred after ubiquitination of RelA, paralleling the nucleolar translocation of the protein (Fig. 3B). Annexin V apoptosis assays showed that this nucleolar translocation of RelA was also associated with induction of apoptosis (Supplementary Fig. S1B). Furthermore, we found that translocation of RelA to the nucleus was required for the apoptotic response to these agents, as this response was completely blocked in cells expressing GFP-RelAΔ27–30 (Fig. 4D). Similar results were observed in HeLa cells, indicating that this mechanism of proteasome inhibitor–mediated apoptosis is not restricted to a particular cell type (Supplementary Fig. S2). In contrast to proteasome inhibition, we found that the apoptotic response to TNFα and TRAIL was identical in colorectal cancer cells expressing GFP-RelAWT and GFP-RelAΔ27–30, indicating that this domain of RelA is dispensable for the proapoptotic or antiapoptotic effects of these agents (Supplementary Fig. S3).

COMMD1 regulates the nuclear distribution of RelA. Data presented thus far show a link between ubiquitination and nucleolar translocation of RelA. To determine whether this relationship was causal, we examined the effect of modulating the RelA ubiquitin ligase complex on the nuclear distribution of the protein. Our studies focused on COMMD1 because this is a rate-limiting component of the complex (24). Using a GST-COMMD1 expression vector and immunochemistry, we found that increased expression of COMMD1 promotes aspirin-mediated nucleolar translocation of RelA, as determined by the number of cells in the whole population showing nucleolar RelA (Fig. 5A). We also found that siRNA-mediated knockdown of COMMD1 blocks aspirin-mediated ubiquitination and nucleolar translocation of the protein (Fig. 5B and C). In fact, we found that on knockdown of COMMD1, RelA accumulates in the nucleoplasm in response to aspirin, confirming a role for the ubiquitin ligase complex in the nucleoplasmic to nucleolar translocation of the protein (Fig. 5C). Consistent with a role for the nucleolar translocation of RelA in the apoptotic response to aspirin, Annexin V apoptosis assays indicated that overexpression of COMMD1 promotes aspirin-mediated apoptosis, whereas knockdown of COMMD1 abrogates this effect (Fig. 5D).

RelA interacts with COMMD1 in response to aspirin treatment. Having established a critical role for COMMD1 in nucleolar translocation of RelA, we determined whether aspirin modulates this protein. We found that aspirin induced a time-dependent increase in COMMD1 levels. This increase was after the effect of the agents on proteasome activity and was concomitant with the ubiquitination and nucleoplasmic accumulation of RelA, prior to nucleolar targeting (Figs. 2B and 6A). Furthermore, this aspirin-mediated increase in COMMD1 levels was associated with increased binding to RelA (Fig. 6B).

Recent studies suggest that phosphorylation of RelA at Ser468 is critical for and promotes its ubiquitination...
and degradation (33, 34). However, we found that treatment with the phosphatase inhibitor calyculin A blocked aspirin- and MG132-mediated ubiquitination of RelA despite promoting S468/S536 phosphorylation of the protein (Fig. 6C).

Taken together, these data provide compelling evidence that the effects of aspirin on COMMD1 promote the ubiquitination of RelA and that this acts as a signal for nucleolar translocation of the protein. They also suggest that, contrary...
Figure 4. Amino acids 27–30 are required for ubiquitination and nucleolar translocation of RelA. A, SW480 cells were transfected with the specified plasmids and treated with MG132 (25 μmol/L) or lactacystin (35 μmol/L) for 16 h as above; then the cellular localization of GFP-tagged RelA was determined in live cells using an Axiovert 100 inverted fluorescent microscope (×40; left). Nucleoli are indicated by arrows in phase-contrast images. Right images, fixed cells counterstained with DAPI (DNA stain). Nucleoli are depicted by areas devoid of DAPI staining (magnification, ×63). B, SW480 cells were transfected with the specified GFP plasmids, then either left untreated (Asp. −) or treated with aspirin (10 μmol/L), carrier (MG132 −), or MG132 (25 μmol/L) for 2 h. The levels of native and high molecular weight (Ub) RelA and total protein ubiquitination were determined by Western blot analysis. C, SW480 cells were transfected and treated with MG132 (25 μmol/L) or aspirin (10 mmol/L) as above. The levels of ubiquitinated RelA were determined by anti-ubiquitin immunoprecipitation (IP) followed by anti-RelA Western blot analysis (WB). Membranes were stripped and reprobed to show the effects of the agents on total protein ubiquitination. Right, SW480 cells constitutively expressing GFP-RelA WT or Δ27–30 were transfected with 6His-ubiquitin. Cells were then treated for 2 h with aspirin (10 mmol/L), and ubiquitinated proteins precipitated using Ni-agarose beads. Precipitated proteins were subjected to anti-RelA and anti-His Western blot analysis. D, SW480 cells were transfected with the specified vectors and then treated with MG132 (25 μmol/L) or lactacystin (0–75 μmol/L) for 16 h. Annexin V-biotin staining, with a Texas red-streptavidin conjugate, was used to identify apoptotic cells. The percentage of cells expressing GFP-tagged RelA undergoing apoptosis was determined by fluorescent microscopy in at least 250 transfected cells for each sample. Columns, mean of at least three independent experiments; bars, SE.
Discussion

Here we report the novel observation that RelA is targeted to the nucleolus to induce apoptosis in response to proteasome inhibition and provide convincing evidence that nucleolar shuttling of RelA, and thus the apoptotic response to specific NF-κB stimuli, is regulated by ubiquitination of the protein.

Based on the data presented here and in our previous studies, we propose the model outlined in Fig. 6D. We suggest that in response to aspirin or stress, the NF-κB pathway is stimulated in parallel with partial inhibition of proteasome activity. This proteasome inhibition allows the accumulation of COMMD1, a critical component of the RelA ubiquitin ligase complex, which itself is regulated by proteasomal degradation to other RelA ubiquitination pathways reported, phosphorylation at S468 is not involved in this process.

Figure 5. COMMD1 modulates the nuclear distribution of RelA. A, immunomicrographs (magnification, ×63) showing aspirin (5 mmol/L, 16 h)-mediated nucleolar translocation of RelA in SW480 cells transfected with GST-COMMD1 or control (GST) vector. NT, nontreated. The percentage of cells in the total cell population showing nucleolar RelA (as indicated by colocalization with the nucleolar marker fibrillarin) was determined in at least 200 cells. Columns, mean (n = 4); bars, SE. Inset, anti-COMMD1 immunoblot showing levels of GST-COMMD1 in transfected SW480 cells. B and C, SW480 cells were transfected with control or COMMD1 siRNA (C1/1 and C1/2) and then treated with aspirin (10 mmol/L) for 0 to 8 h. B, anti-RelA immunoblot showing levels of native and ubiquitylated RelA in response to aspirin (10 mmol/L, 2 h) treatment. Native RelA controls for protein loading. Numbers indicate the mean relative intensity (RI) of high molecular weight RelA (compared with native RelA; quantified using ImageJ; n = 3). C, immunocytochemical staining determined the nuclear distribution of RelA. The percentage of cells showing nucleolar RelA was calculated as above. Inset, Western blot analysis determined the levels of COMMD1 with (+) and without (−) aspirin (10 mmol/L, 2 h) treatment. Actin was used as a control for protein loading. D, SW480 cells were transfected as indicated, then either untreated or treated with aspirin (5 mmol/L) for 24 h. Apoptotic cells were visualized using fluorescent microscopy and the percentage of apoptotic cells within the total cell population was calculated. Columns, average fold increase in apoptosis in response to aspirin compared with the equivalent nontreated control (n = 3); bars, SE.
Figure 6. Aspirin modulates the COMMD1-RelA interaction. A, SW480 cells were treated with 10 mmol/L aspirin for the times indicated and COMMD1 protein levels determined by Western blot analysis. Actin was used as a control for protein loading. B, SW480 cells stably expressing GFP-RelA were transfected with either GST-COMMD1 or an empty vector control (Vec-GST). Twenty-four hours after transfection, cells were treated with 10 mmol/L aspirin for the indicated times, and GST-tagged proteins precipitated from whole-cell lysates using glutathione Sepharose (GSH beads). Anti-RelA Western blot analysis (WB) of precipitated proteins confirmed an interaction between COMMD1 and RelA in response to aspirin treatment. Gels were reprobed with GST to examine the levels of precipitated GST-COMMD1 and GST-control. GFP-RelA and GST-COMMD1 levels in input samples are shown. C, SW480 cells were treated with either aspirin (10 mmol/L) or MG132 (25 μmol/L) for 2 h in the presence or absence of calyculin A (50 nmol/L). Anti-RelA immunoblot shows native and ubiquitinated (Ub) protein in whole-cell extracts. The levels of phosphorylated RelA (S468 and S563) were also determined by Western blot analysis. Filamin was used as a control for protein loading. D, model for nucleolar translocation of RelA and NF-κB–regulated apoptosis in colorectal cancer cells. See Discussion for details.
Accumulated COMMD1 binds RelA, promoting RelA ubiquitination, which, under conditions of proteasome inhibition, signals for nuclear translocation of the protein. It is increasingly recognized that the nucleolus acts as a critical regulator of cell growth and death through sequestration of transcription factors and death-related proteins (35). However, the signals that regulate nucleolar shuttling are still poorly understood. Our conclusion that ubiquitination of RelA acts as a signal for transport to the nucleolus comes from the following findings. First, the ability of NF-κB stimuli to induce nuclear translocation of RelA is paralleled by their ability to induce ubiquitination of the protein (Fig. 2). Second, inhibition of proteasome activity induces ubiquitination and nuclear accumulation of RelA (Fig. 3). Third, for all stimuli, ubiquitination of RelA precedes translocation of the protein to nucleoli (Figs. 2 and 3). Fourth, nuclear translocation and ubiquitination of RelA share a common critical domain (Fig. 4). Fifth, binding between RelA and COMMD1 increases before nuclear translocation of the protein (Fig. 6B). Finally, and most convincingly, knocking down the activity of the RelA ubiquitin ligase complex blocks the nucleoplasmic to nucleolar translocation of the protein (Fig. 5). We did consider that RelA may be ubiquitinated and degraded in the nucleolus, which could explain some of our data. However, in the absence of COMMD1, RelA remained in the nucleoplasm, suggesting that the ubiquitination event occurs before nuclear translocation. Although a number of proteins have been shown to translocate to the nucleolus in response to proteasome inhibition, to our knowledge, this is the first demonstration that ubiquitination acts as a nucleolar targeting signal.

Our suggestion that RelA is ubiquitinated in the nucleoplasm is consistent with the findings of Saccani and colleagues (22) who showed that RelA is ubiquitinated and degraded in the nucleus in a DNA-binding–dependent manner, and that this is an important mechanism for switching off NF-κB transcriptional activity. Based on these and the data presented here, we propose that if RelA is ubiquitinated as a consequence of, or in conjunction with, proteasome inhibition, then the protein is targeted to the nucleolus as an alternative means of switching off NF-κB activity(11).

Our model suggests that stress agents/proteasome inhibitors cause repression of NF-κB–driven transcription by inducing nucleolar translocation of RelA. However, it may be argued that it is the ubiquitination of chromatin-bound RelA that causes repression of NF-κB–driven transcription, and not nucleolar translocation per se. Contrary to this argument, we show that at the time point showing maximum MG132-induced ubiquitination of RelA, the agent has no effect on NF-κB transcriptional activity (Fig. 3C). Similarly, there is a delay of 3 to 5 hours between the peak effects of aspirin on ubiquitination of RelA and the effects of the agent on NF-κB–driven transcription (Fig. 2; ref. 11). Furthermore, for both agents, repression of NF-κB–driven transcription parallels the appearance of RelA in nucleoli (Fig. 3C; ref. 11). Therefore, we would suggest that repression of NF-κB–driven transcription in response to these agents is indeed mediated by nucleolar translocation of the protein.

We found that aa27–30 of RelA are required for ubiquitination of the protein, and our data eliminated the possibility that this was due to ubiquitination of the lysine residue within this motif. Another possibility is that deletion of aa27–30 blocks binding of RelA to its ubiquitin ligase complex, which, in addition to COMMD1, includes elongins B and C, Cul2, and the E3 ligase SOCS1 (24). This suggestion is supported by the data of Maine and colleagues (24) showing that the NH2-terminal 180 amino acids of RelA are important for binding to this complex. We have found that deleting this domain does not abolish RelA binding to COMMD1 (data not shown), and we are currently investigating the role of the domain in binding to other critical members of the complex.

Recent studies have identified phosphorylation at S468 as critical for COMMD1-associated ubiquitination and degradation of RelA (33, 34). However, we found that, while promoting S468 phosphorylation, phosphatase inhibition blocked aspirin- and MG132-mediated ubiquitination of RelA (Fig. 6C). Interestingly, we have previously shown that the COOH terminus of RelA (from aa311) is not required for nucleolar translocation of the protein (11). Taken together, these data would suggest that RelA ubiquitination associated with nucleolar translocation of the protein is independent of S468 phosphorylation. These data may indicate that these stress agents mediate ubiquitination of RelA by an alternative COMMD1-associated pathway to that reported.

Taken together, the data presented here identify a novel mechanism for regulating the nuclear distribution of RelA/apoptosis involving COMMD1 and RelA ubiquitination. These data have considerable relevance to cancer prevention and therapy, as well as to the post-induction regulation of RelA/NF-κB.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Ubiquitination and the Nucleolar Translocation of RelA

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