The Mre11 Cellular Protein Is Modified by Conjugation of Both SUMO-1 and SUMO-2/3 during Adenovirus Infection

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Received 18 January 2014; Accepted 9 March 2014; Published 7 April 2014

Academic Editors: M. Magnani and D. A. Ornelles

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The adenoviral type 5 (Ad5) E1B 55 kDa and E4 Orf6 proteins assemble a Cullin 5–E3 ubiquitin (Ub) ligase that targets, among other cellular proteins, p53 and the Mre11-Rad50-Nbs1 (MRN) complex for degradation. The latter is also inhibited by the E4 Orf3 protein, which promotes the recruitment of Mre11 into specific nuclear sites to promote viral DNA replication. The activities associated with the E1B 55 kDa and E4 Orf6 viral proteins depend mostly on the formation of this E3-Ub ligase. However, E1B 55 kDa can also function as an E3-SUMO ligase, suggesting not only that regulation of cellular proteins by these viral early proteins may depend on polyubiquitination and proteasomal degradation but also that SUMOylation of target proteins may play a key role in their activities. Since Mre11 is a target of both the E1B/E4 Orf6 complex and E4 Orf3, we decided to determine whether Mre11 displayed similar properties to those of other cellular targets, in Ad5-infected cells. We have found that during Ad5-infection, Mre11 is modified by SUMO-1 and SUMO-2/3 conjugation. Unexpectedly, SUMOylation of Mre11 is not exclusively dependent on E1B 55 kDa, E4 Orf6, or E4 Orf3, rather it seems to be influenced by a molecular interplay that involves each of these viral early proteins.

1. Introduction

The adenoviral E1B 55 kDa, E4 Orf6, and E4 Orf3 early proteins are necessary to complete an efficient viral replication cycle. E1B 55 kDa and E4 Orf6 form a complex that associates with an adenovirus-infected-cell-specific Cullin 5-containing E3 ubiquitin (Ub) ligase that induces polyubiquitination and degradation of various cellular targets, namely, the tumor suppressor p53, the Mre11-Rad50-Nbs1 (MRN) complex, DNA ligase IV, bloom helicase, ATRX, Tip60, SPOC1, and integrin α3 [1–12]. The activity of the E1B/E4 Orf6 E3-Ub ligase is required for viral late mRNA export [4, 13], and it has been suggested that most activities associated with EIB 55 kDa and E4 Orf6 depend on formation of this E3-Ub ligase [14]. However, during adenovirus infection of normal human cells (human foreskin fibroblasts, HFF), complete degradation of one of these cellular substrates, the p53 protein, is not attained and reduction of its steady state levels initiates only late during infection [15, 16]. Furthermore, the accumulation of p53 does not lead to apoptosis, and efficient viral replication correlates with the EIB 55 kDa-dependent nuclear localization of p53 to viral replication centers where these proteins colocalize [16–18]. An additional key target of the E1B/E4 Orf6 E3-Ub ligase is the DNA double-strand break repair (DSBR) complex, MRN [5, 8, 19]. Inhibition of DSBR is necessary to avoid concatemerization of the viral genome and to promote efficient viral DNA replication [20–24]. These activities also depend on the E4 Orf3 protein, another early gene product that interacts with EIB 55 kDa as well as the isform II of the promyelocytic leukemia protein (PML). Thereby E4 Orf3 initiates the reorganization of PML nuclear bodies (PML NBs) and formation of nuclear sites where the viral genome is replicated and expressed [8, 22, 23, 25–29].
The PML protein is modified by the small ubiquitin-like modifier (SUMO) system, and this modification is critical for the formation and regulation of PML NBs [30–36]. Like PML, many proteins that localize in PML NBs are SUMOylated [31, 36]. The SUMO system is responsible for the modification of a large pool of cellular proteins [30, 32, 37, 38]. Modification of proteins by SUMO-1 or by SUMO-2 or -3, which display a high degree of similarity to each other and form a family distinct from SUMO-1, achieves a diverse range of effects from cellular stress responses to regulating subcellular localization, transcription factor activity, and protein stability [37–41]. SUMO modification is operated by an enzymatic pathway consisting of an E1 activating enzyme (SAE-2/1), a unique E2 conjugating enzyme (Ubc9), and a number of E3 ligases (reviewed in [30]). While Ubc9 is capable of directly modifying substrates through interaction with a SUMO conjugation motif (Y/KXXE, where Y is a large hydrophobic amino acid and X is any amino acid), E3 ligases add specificity and increase the efficiency of the conjugation reaction. In contrast to SUMO-1, both SUMO-2 and -3 possess an N-terminal SUMO conjugation motif (VKTE) that allows their polymerization to form SUMO chains [42, 43], while conjugation of SUMO-1 to a SUMO-2/3 moiety leads to chain termination [44]. An important role of SUMO modification is to protect modified proteins from proteasomal degradation or conversely to promote their polyubiquitination and degradation [40, 42, 45–48].

During adenovirus infection, the EIB 55 kDa protein undergoes SUMO-1 and SUMO-2/3 [49] modification resulting in regulation of its nucleocytoplasmic shuttling and intranuclear targeting [49–53]. Significantly, EIB 55 kDa not only stimulates SUMOylation of p53 in the PML NBs [54] but also functions as an E3 SUMO-1 ligase for this cellular protein; a modification that contributes to relocalization and maximal transcriptional repression of p53 [55].

These observations confirmed that regulation of p53 in Ad5-infected cells is achieved by processes that do not depend solely on its polyubiquitination and degradation [16–18], suggesting that other cellular targets of the early adenovirus proteins may be regulated by SUMOylation. Therefore, we decided to investigate whether Mre11 is modified by SUMO-1 and/or SUMO-2/3 in Ad5-infected cells. We found that during Ad5 infection of nontumor cells, H1299 and HFF cells displayed distinct SUMO-paralogue colocalization in PML NBs. In contrast to p53 [55], Mre11 SUMOylation was not dependent solely on EIB 55 kDa; rather, our data indicate that during adenovirus infection of nontumor cells, Mre11 is modified by the addition of both SUMO-1 and SUMO-2/3 paralogues and that these modifications depend on concerted activities of the EIB 55 kDa, E4 Orf6, and E4 Orf3 proteins.

2. Materials and Methods

2.1. Cells and Viruses. H1299, HeLa, HEK-293, and human foreskin fibroblast (HFF) cells were grown as monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 5% or 10% fetal bovine serum. The EIB 55 kDa mutant virus A143, carrying a linker insertion in the hybrid Ad2/Ad5 E1B gene, has been described previously [57]. The Ad5 mutant Hr6 carries a frameshift mutation in the EIB coding sequence leading to an EIB 55 kDa null mutant virus [58]. The RF6 mutant carries a point mutation that inhibits EIB 55 kDa binding to Mre11 [59]. The Ad5 wild-type dl309 virus [60] and EIB mutants were propagated in monolayers of HeLa and HEK-293 cells, respectively. Viruses were titered by plaque assay on HEK-293 cells as described [61]. The H5pm4149 virus carries four stop codons in the EIB 55 kDa open reading frame and does not express the EIB 55 kDa protein [62]; H5pm4154 does not express the E4 Orf6 protein and H5pm4150 does not express the E4 Orf3 protein [13]. The three latter virus mutants were propagated in 2E2 cells and titered as fluorescent forming units (FFU) in HEK-293 cells [63].

2.2. Antibodies. Primary antibodies specific for Ad5 proteins included the anti-EIB 55 kDa mouse monoclonal antibody (mAb) 2A6 [64], anti-DBP mouse mAb B6-8 [65], anti-E4 Orf6 mouse mAb RSA3 [66], and anti-E4 Orf3 rat mAb 6A11 [67]. Primary antibodies specific for cellular proteins included the anti-Mre11 mouse mAb 12D7 (GTX70212) (GeneTex, Inc), anti-Nbs1 mouse mAb (GTX70222) (GeneTex, Inc), anti-Rad50 mouse mAb (GTX70228) (GeneTex, Inc), anti-Mre11 rabbit polyclonal antibody (pAb) (pNB 100-142) (Novus Biologicals, Ltd., Cambridge, UK), anti-ß-actin mouse mAb AC-15 (Sigma-Aldrich, Inc.), anti-actin mouse mAb AC-40 (Sigma-Aldrich, Inc.), anti-Ubc9 mouse mAb (610748) (BD Transduction Laboratories), anti-SUMO-1 rabbit mAb (4930) (Cell Signaling), anti-SUMO-2/3 rabbit mAb (4971) (Cell Signaling), anti-HA rat mAb (clone 3F10) (Roche Applied Science), and anti-vimentin rabbit pAb (a gift from Pavel Isa).

2.3. Immunoblotting. To analyze the steady-state concentrations of the cellular Mre11 protein, H1299 and HFF cells at approximately 80–90% confluence were infected with Ad5 or the EIB 55 kDa mutants at 30 plaque forming units (PFU)/cell. For immunoblotting, cells were harvested at indicated times after infection, washed with phosphate-buffered saline (PBS), and extracted with 25 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5% (w/v) sodium deoxycholate, 0.5% (v/v) Nonidet P-40 (NP-40), and 1 mM phenylmethylsulfonyl fluoride for 30 min at 4°C. Cell debris was removed by centrifugation at 10,000 g at 4°C for 5 min. The extracts were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and immunoblotting. For immunoblotting equal amounts of total protein were separated by
SDS-PAGE, transferred to nitrocellulose membranes, and incubated as described previously [68]. The bands were visualized by enhanced chemiluminescence as recommended by the manufacturer (Pierce, Thermo Fisher Scientific, Bonn, Germany) on X-ray films (CEA RP, medical X-ray film). Autoradiograms were scanned and cropped using Adobe Photoshop Elements 8.0.

2.4. Immunoprecipitation. To analyze the SUMOylated forms of the Mre11 protein, HFF cells at approximately 80–90% confluence were infected with Ad5 or the EIB 55 kDa mutants at 30 PFU/cell. For immunoprecipitation, all protein extracts were prepared in RIPA buffer (50 mM Tris- HCl/pH 8.0, 150 mM NaCl, 5 mM EDTA, 0.5% (w/v) sodium deoxycholate, 1% (v/v) Nonidet P-40 (NP-40), 1% (v/v) phenylmethylsulfonyl fluoride (PMSF), 0.1% (v/v) aprotenin, 1 mg/mL leupeptin, 1 mg/mL pepstatin, 25 mM iodoacetamide, and 25 mM N-ethylmaleimide) and kept on ice for 30 min. Cell debris was removed by centrifugation at 10,000 g at 4°C for 5 min. For immunoprecipitation (IP) protein A sepharose was added with 1 μg of anti-Mre11 mAb (12D7) (GeneTex, Inc.) for 1 h at 4°C. The antibody-coupled protein A sepharose was added to pansorbin (50 μL/lgsate; CalBiochem, Merck Chemicals Ltd., Nottingham, UK) preclearred cellular extracts and incubated with constant rotation for 2 h at 4°C. Proteins bound to the antibody-coupled protein A sepharose were sedimented by centrifugation, washed three times, boiled for 3 min at 95°C in 2x Laemmli buffer [69], and analyzed by immunoblotting.

2.5. Percentage of Mre11 SUMOylation. To measure in vivo SUMOylation of Mre11 in Ad infected cells, the EpiSeeker Global Protein SUMOylation Assay Kit (Abcam) was used as described by the manufacturer. Briefly, HFF cells were infected with Ad5 wt or the A143, E4 Orf3-, E4 Orf6-, or EIB 55 K-mutants. Cells were harvested at 16 and 28 h p.i. and nuclei were isolated using nonidet NP-40 as described previously [15]. Nuclear extracts were incubated with the anti-Mre11 rabbit pAB (pNB 100-142) (Novus Biologicals, Ltd., Cambridge, UK) in 8-well assay microwell strips, and SUMO was measured with the anti-SUMO antibody included in the kit by colorimetry. Positive and negative controls included in the kit were used. To calculate SUMOylation of Mre11, the OD values from the negative control were subtracted from the values obtained for extracts from infected cells and were compared to the level of mock-infected cells. Values are expressed as the percentage of SUMO-conjugated Mre11 normalized to the values obtained for Ad5 wt-infected cells. The error bars indicate the standard deviation of the duplicates of two independent measurements.

2.6. Plasmid and Transient Transfection. The expression vectors pcDNA3-empty, pcDNA3-E1B 55 kDa wt [70], pcDNA3-HA-Ubc9, pcDNA3-E4 Orf6 wt, and pcDNA3-E4 Orf3 wt were used for the transfection assays. One hour before transfection, the medium was replaced by DMEM without antibiotics. For transient transfection, the transfection solution containing 1 μL DNA (1 μg/μL), 10 μL of 25 kDa linear polyethylenimine (PEI; Polysciences Inc., Eppelheim, Germany), and 100 μL DMEM was incubated for 30 min at room temperature. After application of the solution, cells were incubated for 6 h before replacing the medium with standard culture medium.

2.7. Immunofluorescence. HFF cells grown on coverslips to approximately 90% confluence were mock-infected or infected with Ad5 wt or EIB 55 kDa, E4 Orf6, or E4 Orf3 null mutant viruses. Cells were processed for immunofluorescence as described previously [68]. After application of specific primary antibodies, cells were incubated with secondary antibodies (Alexa-Fluor 488, 568, and 680, Invitrogen). The coverslips were mounted on glass slides in 10% glycerol and samples were examined by optical section microscopy (Apoptem. Zeiss Axiovert 200 M). Images were taken with an Axiocam MRM using Axiovision 3.1 software (Carl Zeiss, Inc.). Images were assembled using Adobe Photoshop Elements 8.0.

3. Results

3.1. The Mre11 Protein Is not Efficiently Degraded and Is Posttranslationally Modified in Ad5-Infected Normal Human Cells. During Ad5-infection of cultured human cells the DNA damage response (DDR) is initially inhibited by the E4 Orf3 protein, which disrupts PML nuclear bodies (PML NBs) and induces the intracellular redistribution of Mre11 to these sites, prior to its targeted polyubiquitination and proteasomal degradation directed by EIB 55 kDa and E4 Orf6 [8, 14, 22–24, 71]. A similar order of events occurs to regulate p53. In addition, this cellular protein is SUMOylated by EIB 55 kDa and relocalized to nuclear structures [54], where p53 and EIB 55 kDa colocalize [16, 55].

In order to investigate if Mre11 is subject to similar processes, we decided to analyze whether degradation of Mre11 is induced in Ad5-infected human foreskin fibroblasts (HFF). Therefore, we analyzed soluble extracts of Ad5-infected HFF cells by immunoblotting. Adenovirus infection reduced the Mre11 protein levels; however, such reductions could only be observed at time points corresponding to the late phase of infection in HFF cells (28 h postinfection (p.i.)) (Figure 1). Since Mre11 degradation requires association of EIB 55 kDa with E4 Orf6, we examined Mre11 protein levels in cells infected with the EIB 55 kDa insertion mutant (A143) and the EIB 55 kDa null mutant Hr6 [58]. The A143 mutant virus carries a small insertion in the EIB 55 kDa coding sequence [57], which impairs binding to the E4 Orf6 protein [72]. A band of 55 kDa corresponding to EIB 55 kDa was detectable in Ad5 wild-type (wt) and A143 infected cells after immunostaining with the anti-EIB 55 kDa monoclonal antibody (mAb) 2A6. Additionally, we observed bands of higher molecular mass that correspond to the previously observed pattern for the SUMOylated forms of EIB 55 kDa [49, 51–53, 73]. In agreement with previous findings [52], absence of the EIB 55 kDa-E4 Orf6 interaction during infection with the A143 mutant led to more abundant EIB 55 kDa-SUMOylation than during Ad5 infection (Figure 1). Higher levels of Mre11...
could be observed in cells infected with the E1B 55kDa mutants compared to the Ad5 wt virus, indicating that, as expected, degradation of the Mre11 protein was impaired by each of the mutations (Figure 1). This effect on Mre11 protein levels was significantly more pronounced late in infection. In agreement with previous reports, we thus observed that degradation of Mre11 in HFF cells depends on formation of the E1B/E4 Orf6 complex. However, a clear difference in the Mre11 protein pattern, which was particularly noticeable for Mre11 in response to Ad5 and A143 infection compared to the mock-infected control, could be observed in these cells (Figure 1). After immunostaining with the anti-Mre11 mAb (12D7), both Ad5 and A143-infected cell lysates showed additional bands with higher molecular mass (approximately 100 kDa and 200 kDa) than the corresponding Mre11 protein band (81 kDa). These high molecular weight bands were present at low levels in Ad5-infected cells and at significantly increased levels in A143-infected cells. The accumulation of slower-migrating bands detected by the anti-Mre11 mAb suggests this protein may be posttranslationally modified in the course of Ad5 infection.

### 3.2. The Mre11 Protein Colocalizes with E4 Orf3 and E1B 55 kDa in Nuclear and Cytoplasmic Structures throughout the Ad5 Replication Cycle.

The inactivation of MRN during the early phase of infection correlates with the E4 Orf3-induced relocalization of Mre11 to nuclear sites adjacent to the 250kDa band (12D7. GeneTex), or actin (AC-40. Sigma). Actin served as a loading control.

![Figure 1: Degradation of Mre11 is not efficiently induced in Ad infected normal human cells. Degradation of the Mre11 cellular protein over the time course of Ad5 infection was analyzed in HFF cells infected with Ad5 and E1B 55 kDa mutants. Cell lysates were prepared at the indicated times postinfection for analysis by Western blot with antibodies directed to E1B 55 kDa (2A6), Mre11 (12D7. GeneTex), or actin (AC-40. Sigma). Actin served as a loading control.](image-url)

In order to determine whether the Mre11 protein is SUMOylated during adenovirus infection, we decided to test the addition of SUMO paralogues to Mre11. To evaluate whether the protein can be modified by SUMO-1 and SUMO-2/3 we performed immunoprecipitation assays at different times postinfection (10, 16, 28, and 36 h p.i.) from extracts of cells infected with Ad5, the E1B 55 kDa insertion mutant A143, or virus mutants that do not express either E1B 55 kDa, E4 Orf6, or E4 Orf3. For the pull down assays anti-Mre11 was used, and subsequent Western blot analyses were performed using monoclonal antibodies directed specifically to SUMO-1 or SUMO-2/3 (Figure 3(a)) thus allowing us to distinguish SUMO-1 from SUMO-2/3 paralogues.

Immunoprecipitated Mre11 and subsequent SUMO-2/3 immunostaining showed bands with molecular masses of approximately 130 kDa, 180 kDa, and 250 kDa, which correspond to different forms of poly-SUMO-2/3 chains covalently attached to Mre11. As shown in Figure 3(a), the pattern of SUMO-2/3-modified Mre11 bands was very dynamic throughout the Ad5 replication cycle. The SUMO-2/3 forms of Mre11 were present from the early phase (10 and 16 h p.i.) and remained well into the late phase (28 and 36 h p.i.), in agreement with the sustained nuclear Mre11 signal observed by immunofluorescence analysis (Figure 2). Interestingly, different patterns were observed between the Ad5 and mutant viruses. The 130 kDa band was the most abundant form of SUMO-2/3-Mre11 detected during infection with either Ad5 or A143 (Figure 3(a)), where this band was detected throughout infection. In contrast to Ad5-infected cells, where the 250 kDa band was only observed late during infection (36 h p.i.), in A143-infected cells this band was present from...
the early phase of the viral replication cycle (16 h p.i.). Throughout infection, this 250 kDa band was significantly more abundant in cells infected with the E1B 55 kDa- or E4 Orf3 null mutants. In addition a 180 kDa band which was observed in cells infected with A143, as well as with the E1B 55 kDa- or E4 Orf3-minus mutant viruses, was not detected during infection with Ad5 wt (Figure 3(a)). A clear difference in the pattern and a higher overall abundance of SUMOylated bands was observed in cells infected with the E4 Orf6 null mutant, where multiple SUMO-2/3 forms of Mre11 were detected and a band with a molecular weight higher than 250 kDa was prominent. As control for total Mre11 input levels the same samples were also analyzed by Western blot using the anti-Mre11 (Mre11 long exposure). These results clearly show that Mre11 is modified by addition of SUMO-2/3, in agreement with recently published results [56]. However, these data also demonstrate that during infection the SUMO-2/3 modification of Mre11 can occur in the absence of either E1B 55 kDa, E4 Orf6, or E4 Orf3. Nevertheless, since we observed a clear difference in the pattern of Mre11-SUMO-2/3 bands between Ad5 and the different mutant viruses (Figure 3(a)), these data suggest that all three proteins have the ability to modulate SUMOylation of Mre11 in the context of infection. We then decided to test the addition of SUMO-1 to Mre11. In mock-infected cells, we observed a 110 kDa band that corresponded to SUMO-1-modified Mre11 (Figure 3(a)). The SUMO-1-modified Mre11 was also clearly visible during infection with the Ad5, A143, or the E1B 55 kDa, E4 Orf6, or E4 Orf3 null mutant viruses. However, during Ad5 infection, the level of SUMO-1-modified Mre11 decreased as the viral replication cycle progressed, displaying significantly reduced levels by 36 h p.i. Such reduction of SUMO-1-Mre11 levels was not observed in cells infected with any of the mutant viruses; rather, the level of SUMO-1-Mre11 increased by 36 h p.i. in the absence of E1B or E4 Orf3. These data indicate that Mre11 can be modified by SUMO-1 and furthermore suggest that during adenovirus infection the E1B 55 K, E4 Orf6, and E4 Orf3 proteins can each modulate SUMO-1 addition to Mre11.

In order to further explore the relative impact of EIB 55 kDa, E4 Orf3, E4 Orf6, and the EIB/E4 Orf6 complex on SUMOylation of Mre11 during virus infection, we decided to measure total levels of Mre11 SUMOylation, detecting SUMO-1 as well as SUMO-2 and -3-modified forms, at different times postinfection (16 and 28 h p.i.). Using nuclear extracts of infected HFF cells, anti-Mre11 and anti-SUMO antibodies were used to measure the modified form of Mre11 through the signal reporter-color development system, as described in Section 2. As expected, in Ad5-infected cells,
Figure 3: The Mre11 protein is modified by addition of both SUMO-1 and SUMO-2/3 during Ad5 infection. (a) HFF cells either mock-infected or infected with Ad5, the E1B 55kDa insertion mutant A143, or E1B 55kDa, E4 Orf6, or E4 Orf3 null mutants were harvested at different times postinfection (10, 16, 28, and 36 h p.i.), and lysates were analyzed by immunoprecipitation and Western blot. The immunoprecipitation of Mre11 was performed using anti-Mre11, resolved on 10% SDS-PAGE, and visualized by immunoblotting using anti-SUMO-2/3 or anti-SUMO-1 mAbs. Specific Mre11-SUMO-2/3 or -SUMO-1 bands are indicated by (∗). Western blotting was performed using anti-Mre11 as input control. DBP and actin were included as infection control and internal loading control, respectively. (b) Quantification of total SUMO-Mre11 protein. Nuclear extracts of HFF cells either mock-infected or infected with Ad5, A143, or virus null mutants (E1B 55kDa, E4 Orf6, or E4 Orf3) were obtained at two different times postinfection (16 and 28 h p.i.). Nuclear extracts were incubated with the anti-Mre11, and SUMO was detected by a colorimetric assay, as described in Section 2.

we observed approximately 20% decrease in the total level of SUMO-modified Mre11 in the late phase of infection compared with the early phase (Figure 3(b)). This is consistent with the lower levels of SUMO-1-Mre11, which are more prominent in Ad5 infection than the SUMO-2 and -3 modified protein, detected at 36 h p.i. (Figure 3(a)) and with the degradation of Mre11 induced during the late phase of the replication cycle. To compare the relative effect of E1B 55kDa, E4 Orf3, E4 Orf6, and the E1B/E4 Orf6 complex, the SUMO-Mre11 values obtained for each mutant virus were normalized to Ad5 levels, which were set to 100% at 16 h p.i. (Figure 3(b)). Lower levels of SUMOylated Mre11 were observed in the A143, E4 Orf6, and E4 Orf3 mutants at 16 h p.i. Interestingly, in contrast to the decrease observed for Ad5, during A143 infection, when the E1B/E4 Orf6 E3-Ub complex does not assemble and Mre11 degradation is not induced, we observed a slight increase in the level of total SUMO-Mre11 levels in the late phase versus the early phase of infection. A similar increase was observed during infection with the E1B- or E4 Orf6-null mutants. In contrast to Ad5, during infection with the E4 Orf3 null mutant, the levels of SUMO-Mre11 displayed a marked decrease at 16 h p.i.; however, by 28 h p.i. SUMO-Mre11 levels were comparable to those of Ad5. These data suggest that while E4 Orf3 may be necessary to induce SUMO addition to Mre11 during the early phase of infection, it is dispensable during the late phase, when EIB 55kDa and E4 Orf6 seem to have a compensatory effect in the SUMOylation of Mre11
3.4. The E1B 55 kDa, E4 Orf6, and E4 Orf3 Proteins Can Each Modulate Mre11 SUMOylation during Adenovirus Infection.

To further investigate the role of E1B 55 kDa, E4 Orf6, or E4 Orf3 in Mre11-SUMOylation, we decided to determine whether any one of these viral early proteins was sufficient to induce SUMO addition to Mre11 independent of an adenoviral infection environment. We overexpressed each of these viral proteins independently (E1B 55 kDa, E4 Orf6, and E4 Orf3) in Mre11-SUMOylation, and in combination with Ubc9. For these experiments we transfected H1299 cells with either an empty vector or a plasmid encoding HA-Ubc9 as controls or plasmids encoding E1B 55 kDa, E4 Orf6, or E4 Orf3 plus the HA-Ubc9 plasmid. The soluble extracts of the transfected cells were analyzed by Western blot (Figure 4(a)). Significantly, when we transfected an empty pcDNA plasmid or pcDNA in combination with the HA-Ubc9 plasmid, as controls, we observed a higher molecular weight band of approximately 180 kDa, matching the molecular mass of one of the SUMO-2/3-Mre11 bands observed in previous experiments (Figure 3(a)). This observation suggested that the stress caused by the transfection assays was enough to induce formation of the 180 kDa form of SUMO-2/3-Mre11. When each of the viral early proteins was overexpressed in combination with the HA-Ubc9 protein, we detected an additional band of higher molecular mass of approximately 250 kDa (Figure 4(b)). No differences in the pattern of bands corresponding to SUMO-2/3-Mre11 were observed between cells transfected with any of the three viral early proteins, suggesting that outside the context of virus infection and when Ubc9 is overexpressed E1B 55 kDa, E4 Orf6, and E4 Orf3 can each induce addition of SUMO-2/3 to Mre11 to form the 250 kDa SUMO-2/3-modified Mre11 independently of one another.

As described in the introduction, the EIB 55 kDa protein can function as an E3 SUMO-ligase for p53, and it provides target specificity to the Cullin5-E3 ubiquitin ligase inducing degradation of p53 and other targets, including Mre11. To explore whether the interaction between EIB 55 kDa and Mre11 may be directly implicated in its SUMOylation, we analyzed the SUMO status of Mre11 in cells infected with the RF6 mutant, which carries a point mutation that abrogates binding of EIB 55 kDa to Mre11 [59, 75]. H1299 cells
were infected with Ad5, the E1B 55 kDa null, or the RF6 mutant virus, and infected-cell extracts were analyzed by Western blot (Figure 4(b)). As expected, both E1B 55 kDa virus mutants failed to induce decreased levels of Mre11 compared to Ad5 wt, since they either do not form the E3-Ub ligase (E1B-) or cannot recruit Mre11 for ubiquitination (RF6) [53, 76]. Interestingly, the patterns of bands observed during the early and late phase of infection were different between the Ad5 wt and E1B 55 kDa mutants. At 16 h p.i. two high molecular mass bands of approximately 180 kDa and 250 kDa were observed in extracts of cells infected with either of the E1B 55 kDa mutants. In contrast, during late infection with each of the E1B 55 kDa mutants (28 h p.i.), the 110 kDa form of Mre11 accumulated to higher levels than in Ad5 wt infection, indicating that the levels of SUMO-1 modified Mre11 increased during the late phase of infection in the absence of E1B 55 kDa protein or the E1B 55 kDa-Mre11 interaction. In addition, during late infection with RF6 only the 110 kDa band was visible; infection with the E1B 55 kDa null mutant resulted in the appearance of both the 110 kDa and 130 kDa bands that correspond to SUMO-1 and SUMO-2/3 forms of Mre11, respectively (Figure 4(b)). These results suggest that a different SUMOylation pattern of Mre11 was induced in the absence of E1B 55 kDa and that the E1B 55 kDa-Mre11 interaction may be necessary for SUMO-2/3 addition to Mre11 only in the late phase of infection. Taken together these data indicate that although E1B 55 kDa is not absolutely required to induce SUMOylation of Mre11 in infected cells, it can influence the level and pattern of SUMO addition to this cellular protein at different times during the viral replication cycle.

3.5. Ad5-Infection Induces Reorganization of SUMO-1 and SUMO-2/3 Paralogues. SUMOylation of PML plays an important role for the scaffold function of this protein in functional PML NB organization. Furthermore, SUMO-1 or SUMO-2/3 attachment to PML is responsible for the correct localization of PML in the PML NBs [36]. SUMO paralogues display distinct localization in the PML NBs when analyzed by immunofluorescence microscopy. While SUMO-1 modified proteins are located in the periphery of PML NBs forming a shell-like appearance, SUMO-2/3 proteins seem to occupy the interior of these structures [33]. We decided to investigate whether Ad5-infection induces the nuclear redistribution of SUMO-1 and SUMO-2/3. We therefore performed immunofluorescence analysis of the intracellular distribution of SUMO-1, SUMO-2/3, Mre11, and E4 Orf3 in HFF cells infected with Ad5 wt, A143, or the EIB 55 kDa, E4 Orf6, or E4 Orf3 viruses, at 28 h p.i. DBP is shown in red and SUMO-2/3 in green; DAPI-stained nuclei are shown in blue. 

Figure 5: Ad5-infection induces the nuclear reorganization of SUMO-1 and SUMO-2/3 paralogues. (a) The intracellular distribution of E4 Orf3, Mre11, and SUMO-1 was analyzed in HFF cells infected with Ad5, the A143, or the EIB 55 kDa, E4 Orf6, or E4 Orf3 null mutants at 28 h p.i. After infection cells were processed for immunofluorescence as described in Section 2. E4 Orf3 is shown in magenta, Mre11 in red, and SUMO-1 in green. (b) The intracellular distribution of E4 Orf3 and SUMO-2/3 was analyzed in HFF cells infected with Ad5, A143, or the null mutants for EIB 55 kDa, E4 Orf6, or E4 Orf3. E4 Orf3 is shown in red and SUMO-2/3 in green; DAPI-stained nuclei are shown in blue. Insets were enlarged 5X to show the localization of SUMO-2/3 in ring-like structures, adjacent to E4 Orf3 tracks. (c) The intracellular distribution of DBP and SUMO-2/3 was analyzed in HFF cells infected with the Ad5, A143, or the null mutants for EIB 55 kDa or E4 Orf3 viruses, at 28 h p.i. DBP is shown in red and SUMO-2/3 in green; DAPI-stained nuclei are shown in blue.
cells to speck- or track-like structures in the cell nucleus of Ad5 wt-infected HFF (90% of all cells examined, n > 60). The redistribution of SUMO-1 seemed to be independent of the association between E1B 55 kDa and E4 Orf6 or the presence of either of these viral proteins, since it was also induced in cells infected with the A143, E1B 55 kDa-, or E4 Orf6-mutant virus (Figure 5(a)). As expected Mre11 colocalized with E4 Orf3 [27], and the staining pattern of both of these proteins overlapped with SUMO-1 in the majority of nuclear track-like structures in cells infected with either Ad5 wt, A143, E1B 55 kDa-null, or E4 Orf6-null mutants (90% of all cells examined, n > 60) (Figure 5(a)). During infection with the E4 Orf3 null mutant, however, we found that neither Mre11 nor SUMO-1 was reorganized into nuclear tracks but relocalized to large dot-like structures showing a stronger Mre11 and SUMO-1 staining pattern. These data demonstrate that while E1B 55 kDa and E4 Orf6 are dispensable, E4 Orf3 is necessary for the nuclear reorganization of both Mre11 and SUMO-1 to tracks, consistent with previous observations [23, 27, 56, 71].

We next analyzed the intracellular distribution of SUMO-2/3 during infection with Ad5 and the viral mutants using the same setup as before (Figures 5(b) and 5(c)). In mock-infected cells, we observed a diffuse SUMO-2/3 distribution throughout the nucleoplasm, with small dots displaying a slightly stronger signal. In Ad5 wt-infected cells, the SUMO-2/3 signal was reorganized into a reticular distribution where the protein accumulated in ring-like spherical structures (Figures 5(b)-insets and 5(c)). These structures were closely associated and displayed partial colocalization with adjacent E Orf3 tracks (80% of all cells examined, n > 30) (Figure 5(b)). Additionally, this distribution of SUMO-2/3 was similar to viral replication centers marked by the E2 DNA-binding protein (DBP) (Figure 5(c)), which was additionally juxtaposed with E4 Orf3 (Figure 5(b)) or PML tracks (not shown). During infection with the EIB 55 kDa or E4 Orf6 null mutants, SUMO-2/3 was reorganized into nuclear tracks and colocalized with E4 Orf3 (Figure 5(b)). In contrast, during infection with the A143 or the E4 Orf3 null mutant viruses, SUMO-2/3 colocalized more extensively with DBP (Figure 5(c)). Interestingly, relocation of SUMO-2/3 to sites of viral DNA replication (marked by DBP) was enhanced in the absence of a functional EIB/E4 Orf6 complex (A143) or the absence of E Orf3 (Figure 5(c)). These data indicate that in contrast to SUMO-1, whose reorganization and colocalization depends on E4 Orf3, the extensive relocation of SUMO-2/3 to viral replication centers is dependent on the presence of both EIB 55 kDa and E4 Orf6 (Figures 5(b) and 5(c)). Altogether, these observations suggest a compensatory effect between the three viral proteins in the modulation of the SUMO paralogues during infection. Moreover, the localization of SUMO-1 was influenced by E4 Orf3 and unaffected by EIB 55 kDa or E4 Orf6, while SUMO-2/3 relocation to viral replication centers was dependent on EIB 55 kDa and E4 Orf6. These results suggest that a complex molecular interplay between the EIB 55 kDa, E4 Orf6, and E4 Orf3 proteins is responsible for the concerted regulation and modification of protein targets with the different SUMO paralogues during adenovirus infection.

### 4. Discussion

Various components of the DDR are regulated by posttranslational modifications, including phosphorylation, ubiquitylation, acetylation, methylation, NEDDylation, and SUMOylation [77]. Following DNA damage, several DNA repair factors transit to and from PML NBs, and the PML NBs themselves colocalize with sites of unscheduled DNA synthesis [78–80]. In the case of Mre11, its phosphorylation and methylation have been studied in detail [81–87], and the protein is known to shuttle to PML NBs in response to DNA damage, in an arginine methylation-dependent manner [86, 88]. However, although Mre11 is predicted to possess up to two potential SUMOylation sites, the role of this modification in the regulation of the protein's activities is not known [80, 89].

Here we have shown that Mre11 is modified by both SUMO-1 and SUMO-2/3 during infection with Ad5. The SUMOylated forms of Mre11 reproducibly displayed a complex pattern of well-defined bands in Western blot analyses that varied depending on the presence of the EIB 55 kDa, E4 Orf6, or E4 Orf3 proteins and on the ability of EIB 55 kDa to interact with E4 Orf6 or Mre11. The pulldown of Mre11 and the use of antibodies directed against either the SUMO-1 or SUMO-2/3 paralogues yielded bands with approximate molecular masses of 110, 130, 180, and 250 kDa. The 110 kDa band was observed only with antibodies directed against SUMO-1, suggesting that Mre11 was monoSUMOylated with this paralogue during infection. Prediction of potential SUMOylation sites in the Mre11 sequence has shown that there exist two putative lysines within consensus SUMO motifs (lysines 255 and 661) [90]. We do not yet know whether only one putative lysine or each lysine may be modified, with the latter resulting in a combined population of Mre11 proteins monoSUMOylated in different lysines. In contrast, higher molecular weight bands were observed only with the anti-SUMO-2/3 antibody, indicating that modification by both mono- and poly-SUMO-2/3 can occur either on one or more lysines.

As described in the introduction, EIB 55 kDa, E4 Orf6, and E4 Orf3 are required to inhibit the antiviral cellular defenses and to promote the expression of viral late genes, resulting in efficient viral replication [91]. Two key processes they modulate are the relocalization and the proteasomal degradation of cellular targets that would otherwise interfere with viral replication [2, 7–9, 14, 22, 23]. Interestingly, these viral early proteins inhibit an overlapping, but nonidentical, set of cellular target proteins. All three participate in degradation of cellular targets. Protein ubiquitination by EIB 55 kDa and E4 Orf6 depends on Cul5 or Cul2 containing E3-ubiquitin ligases whose assembly is directed by E4 Orf6, while EIB 55 kDa provides specificity for substrate recognition [2, 92]. In addition, EIB 55 kDa can induce degradation of the cellular transcription repressor, Daxx, independently of E4 Orf6 [93], and the E4 Orf3 protein can mediate turnover of the transcriptional intermediary factor 1p (TIF 1p), in the absence of either E4 Orf6 or EIB 55 kDa [94], suggesting that each can modulate ubiquitination of cellular targets by both common and independent mechanisms. Interestingly, EIB 55 kDa and E4 Orf3 can also modulate the SUMOylation
machinery, suggesting that regulation of cellular proteins by these viral gene products is likely to be influenced by a crosstalk between ubiquitination and SUMOylation. The data presented here now show that addition of SUMO to Mre11 during adenoviral infection is highly dynamic. The absence of E1B 55 kDa, E4 Orf6, or E4 Orf3, when neither degradation (by the E1B/E4 Orf6 complex) nor relocalization (by E4 Orf3) of Mre11 can be induced, led to the accumulation of slower migrating SUMO-modified Mre11 forms already early during infection and increased as infection progressed. However, infection with the Ad5 and each of the virus mutants produced different patterns of SUMOylated Mre11 bands, indicating that each of these proteins can differentially modulate the cellular SUMOylation system.

During infection, different adenoviral proteins modulate the cellular SUMO machinery. In the early phase, ElA interacts with Ubc9 interfering with polySUMOylation of cellular substrates, while monoSUMOylation is unaffected [95]. In the case of EIB 55 kDa, the protein is targeted by both, SUMO-1 and SUMO-2/3 addition [73], and in addition the EIB 55 kDa protein can act as an E3-SUMO-1-ligase for p53 [35]. EIB 55 kDa also interacts with Ubc9, and the degradation of p53 and Mre11 are both independent of the SUMOylation or phosphorylation status of EIB 55 kDa [49]. The SUMOylated form of EIB 55 kDa seems to be associated with an insoluble nuclear matrix fraction early during infection, when the protein associates with PML isoform IV in the PML NBs [52]. EIB 55 kDa can also interact with E4 Orf3 favoring its association with PML [26]. Upon formation of the EIB/E4 Orf6 complex E4 Orf6 has been suggested to displace EIB 55 kDa from E4 Orf3 and from the insoluble fraction, resulting in a soluble complex that associates with viral replication centers or the Cul5-E3-Ubiquitin ligase [49, 52]. A crucial player in the reorganization of PML NBs during infection is the E4 Orf3 protein, which can also impact the SUMOylation machinery [56]. Intriguingly, our results have shown that SUMOylation of Mre11 is influenced by the EIB 55 kDa, E4 Orf6, and E4 Orf3 proteins. Transient expression of each of these viral early proteins was sufficient to induce SUMO addition to Mre11, while in the context of a viral infection none of them was absolutely required for Mre11 SUMOylation. Rather, the pattern of SUMOylated bands observed during the early and late phases of infection indicated that a temporal regulation of SUMO addition was influenced by a dynamic molecular interplay that involves each of these viral early proteins.

Our data showed that, as expected, the relocalization of Mre11 to E4 Orf3 and PML containing tracks (Figure 2; data not shown) was independent of EIB 55 kDa or E4 Orf6 or formation of the EIB/E4 Orf6 complex (Figures 2 and 5(a)) and that E4 Orf3 was necessary to induce the relocalization of both Mre11 and SUMO-1 to PML tracks in infected cells (Figure 5(a)). Intriguingly, the colocalization of E4 Orf3 with SUMO-2/3 was not as abundant, as the latter accumulated mostly in ring-like spherical structures that colocalized with DBP in viral replication centers (Figure 5(c)). The distribution of SUMO-1 and SUMO-2/3 associated to PML NBs is highly dynamic but has been shown to display distinct patterns, where SUMO-1 appears as a spherical shell that surrounds PML NBs and colocalizes with PML and SP100, while SUMO-2/3 can be located in the interior of the spherical PML NBs [33]. Our findings therefore suggest that SUMO-1 may be retained in the E4 Orf3-reorganized PML tracks that form adjacent to the viral replication centers, while SUMO-2/3 colocalizes only partially with these tracks and is instead mostly relocated to sites of viral DNA replication. The colocalization of SUMO-2/3 with DBP in these spherical structures suggests that SUMO-2/3 may be localized to the interior of the viral replication centers where it could function as a scaffold as it does in the PML NBs [33]. The more extensive recruitment of SUMO-2/3 to the interior of viral replication centers occurred only in the absence of E4 Orf3 or in the presence of both EIB 55 kDa and E4 Orf6, regardless of whether the EIB/E4 Orf6 complex can form, suggesting that each of these proteins is required for the concerted recruitment of SUMO-2/3 to the viral replication centers. These findings suggest that these three viral early proteins modulate the relocalization of SUMO paralogues to counteract antiviral host defenses that are coordinated by the PML NBs. Since other components of DDR are known to be modified by SUMO [77], it will be interesting to determine whether the different SUMO paralogues are used in Ad infected cells to modify and regulate specific DDR proteins in the PML NBs or viral replication centers.

The SUMO-modified forms of EIB 55 kDa were also more readily observed with the A143 mutant than with the Ad5 wt (Figure 1). This was in agreement with previous findings showing that EIB 55 kDa SUMOylation increases in the absence of E4 Orf6, when association of EIB 55 kDa with E4 Orf3 and PML NBs is enhanced [52]. Interestingly, in the absence of the E3-Ub ligase, the SUMO-modified forms of both EIB 55 kDa and Mre11 accumulated to a higher extent during the late phase of infection. Taken together these findings indicate that the SUMOylated forms of Mre11 increase when degradation of the protein is precluded, suggesting that SUMOylation may be induced as a cellular mechanism that protects Mre11 from degradation. An alternative possibility is that addition of SUMO-2/3 modulates Mre11 polyubiquitination. Interestingly, while SUMO-1 addition does not correlate extensively with polyubiquitination and protein degradation, SUMO-2-conjugated proteins are more commonly ubiquitylated, linking SUMO-2 modification to the ubiquitin-proteasome system [42]. In our experiments, the pattern of SUMO-modified Mre11 bands changed as viral replication progressed. While during infection with Ad5, when Mre11 degradation can be induced, slower migrating bands corresponding to the highest SUMO-2/3 modified Mre11 forms were noticeable at early times postinfection; these bands decreased by the late phase correlating with the decrease in Mre11 levels. In contrast, these bands increased in intensity in the absence of EIB 55 kDa or when EIB 55 kDa cannot interact with Mre11 (RF6), when degradation of Mre11 cannot be induced (Figure 4(b)). Interestingly at late times, the pattern of multi-SUMO-modified Mre11 switched from the slower migrating 180 and 250 kDa bands to the faster migrating, 110 and 130 kDa bands, which correspond to monoSUMO-1 and monoSUMO-2/3, respectively (Figure 4(b)). Therefore, it will be interesting to investigate whether EIB 55 kDa, E4 Orf6, and
E4 Orf3 may be responsible for the temporal regulation of the SUMO-ubiquitin switch.

Sohn and Hearing recently reported [56] that Ad infection induces modification of Mre11 by SUMO-2 conjugation and of Nbs1 by both SUMO-1 and -2 in HeLa cells. In their experiments E4 Orf3 was shown to be necessary and sufficient for this modification, while deconjugation of SUMO from Nbs1 and not Mre11 was influenced by EIB 55 kDa or E4 Orf6 [56]. Our results now confirm that Mre11 SUMOylation is induced in Ad infected normal HFF and in transformed H1299 cells. However, we have demonstrated that Mre11 is also modified by the addition of SUMO-1. The reason for the difference in the SUMO-1 modification of Mre11 is not clear and should be explored to determine whether it may depend on experimental conditions. However, while Sohn and Hearing [56] used transfected HeLa cells and overexpression of both SUMO paralogues and E4 Orf3, in our experiments SUMO-1 conjugation to Mre11 was observed in normal human foreskin fibroblast infected cells. More importantly our results now show that EIB 55 kDa and the EIB/E4 Orf6 complex influence the redistribution of SUMO-2/3 in the cell nucleus as well as SUMOylation of Mre11. Therefore, these data indicate for the first time that each of these proteins can modulate SUMO modification or degradation of cellular substrates underscoring the complexity of the molecular interplay that underlies the activities of the EIB 55 kDa, E4 Orf6, and E4 Orf3 proteins in regulation of SUMOylation and ubiquitination during Ad infection.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

The authors thank Pavel Isa for monoclonal antivimentin and Arnie Berk for the EIB 55 kDa insertion mutant A143. This work was supported by grants from CONACyT-SEP (SEP-2008-84582; CB-2011-01-168497) and Promep-SEP for R.A.G. The Heinrich Pette Institute is supported by the Freie und Hansestadt Hamburg and the Bundesministerium für Gesundheit (BMG). Ramon A. Gonzalez and Thomas Dobner received support from the Research Group Linkage Program of the Alexander von Humboldt Foundation. Elizabeth Castillo-Villanueva and Paloma Hidalgo received scholarships from CONACyT (nos. 215337 and 447442, resp.). Sabrina Schreiner is supported by the Peter and Traudl Engelhorn Stiftung. This work is part of the PhD dissertation submitted by Elizabeth Castillo-Villanueva in partial fulfillment of the degree requirements.

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