High Avidity Binding to DNA Protects Ubiquitylated Substrates from Proteasomal Degradation*

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Giuseppe Coppotelli, Nouman Mughal, Diego Marescotti1, and Maria G. Masucci2

From the Department of Cell and Molecular Biology, Karolinska Institutet, 17177 Stockholm, Sweden

Protein domains that act as degradation and stabilization signals regulate the rate of turnover of proteasomal substrates. Here we report that the bipartite Gly-Arg repeat of the Epstein-Barr virus (EBV) nuclear antigen (EBNA)-1 acts as a stabilization signal that inhibits proteasomal degradation in the nucleus by promoting binding to cellular DNA. Protection can be transferred by grafting the domain to unrelated proteasomal substrates and does not involve changes of ubiquitylation. Protection is also afforded by other protein domains that, similar to the Gly-Arg repeat, mediate high avidity binding to DNA, as exemplified by resistance to detergent extraction. Our findings identify high avidity binding to DNA as a portable inhibitory signal that counteracts proteasomal degradation.

The ubiquitin-proteasome system controls the turnover of regulatory proteins and the disposal of aberrant polypeptides (1). The efficiency of proteasomal degradation is regulated by at least three features of the substrate (2). Constitutive or conditional degradation signals act as recruitment sites for ubiquitin ligases that mediate the conjugation of polyubiquitin tags that target the substrate to the proteasome (3). In addition, efficient degradation requires the presence of unstructured domains of discrete length that anchor the substrate to the proteasome and serve as initiation sites for unfolding and translocation in the catalytic chamber of the protease (4, 5). Finally, proteolysis is regulated by signals that delay or block the activity of the proteasome. Similar to degradation signals, stabilization signals are modular domains whose removal accelerates protein turnover, whereas their grafting to unrelated proteasome substrates results in complete or partial inhibition of proteolysis (6). The regulation of different steps of the proteolytic process by modular domains can be exploited to control proteolysis in a space- and time-dependent manner, as recently illustrated by the use of a conditional mutant Escherichia coli dihydrofolate reductase (ecDHFR) degradation signal that is inactivated by the small-molecule ligand trimethoprim (7).

The Gly-Ala repeat (GAr)3 of the Epstein-Barr virus (EBV) nuclear antigen-1 (EBNA1) is the first recognized example of a cis-acting domain serving as a transferable stabilization signal (8, 9). GAr domains of different length prevent the degradation of a variety of proteasomal substrates including IκB (10), p53 (11), and an N-degron bearing GFP (12), and grafting of the GAr was successfully exploited to generate immune stealth variants of LacZ, firely luciferase, and the HSV1 thymidine kinase that can evade rejection in gene therapy settings (13, 14). The GAr does not prevent ubiquitylation (10, 11) but alters the interaction of ubiquitylated substrates with the proteasome (10, 15, 16), which causes premature release and, depending on the substrate, complete or partial inhibition of proteolysis. In addition to the GAr, other features of EBNA1 regulate its degradation by the proteasome because a GAr-deleted protein remains relatively stable (17, 18). We have addressed this issue by monitoring the turnover of wild type and GAr-deleted EBNA1 (EBNA1-ΔGA) in different cellular compartments. We found that in the absence of the GAr, EBNA1 is stable in the nucleus but is efficiently degraded by the proteasome in the cytoplasm. The stability of nuclear EBNA1 was dependent on the presence of a bipartite Gly-Arg repeat (GRr) domain that tethers the protein to cellular DNA. Grafting the GRr or other protein domains that confer detergent-resistant binding to DNA inhibited the degradation of a short-lived proteasomal substrate without interfering with ubiquitylation. Thus, our findings identify high avidity binding to DNA as a portable inhibitory signal that counteracts proteasomal degradation.

EXPERIMENTAL PROCEDURES

Reagents—Dulbecco’s modified Eagle’s medium (DMEM), RPMI 1640 medium, penicillin-streptomycin, L-glutamine, cycloheximide (CHX), doxycycline (Dox), Tween®20, Triton X-100, bovine serum albumin (BSA), dithiothreitol (DTT), IGEPAL CA-630, EDTA, and puromycin were purchased from Sigma-Aldrich. Fetal bovine serum (FBS) and Genetix 418 (G418) were purchased from Invitrogen. Formaldehyde solution 37% was from Merck (Darmstadt, Germany). MG132 and epoxomicin were purchased from Enzo Life Sciences (Exeter, UK). Hygromycin B was from Calbiochem. Complete protease inhibitors were from Roche Applied Science.

1 The abbreviations used are: GAr, EBNA1 Gly-Ala repeat; GRr, EBNA1 Gly-Arg repeat; EBV, Epstein-Barr virus; EBNA1, EBV nuclear antigen-1, CHX, cycloheximide; Dox, doxycycline; DBD, DNA binding domain; VK, variable κ chain; NLS, nuclear localization signal; Bis-Tris, 2-(bis(2-hydroxyethyl)-amino)-2-(hydroxymethyl)propane-1,3-diol; TRITC, tetramethylrhodamine isothiocyanate.

2 To whom correspondence should be addressed: Dept. of Cell and Molecular Biology, Karolinska Institutet, Box 285, 17177 Stockholm, Sweden. E-mail: maria.masucci@ki.se.
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Antibodies—Mouse monoclonal antibodies anti-FLAG® (M2, working dilution 1:10000) and anti-β-actin (AC-15, 1:5000) were from Sigma-Aldrich. The anti-EBNA1 mouse monoclonal OT-1x (1:2000) and affinity-purified rabbit anti-peptide serum K67.3 (1:1000) were a gift from J. Middeldorp, Vrije Universiteit, Amsterdam. Polyclonal rabbit anti-ubiquitin (1:1000) was from DAKO (Glostrup, Denmark). Polyclonal rabbit anti-ubiquitin (1:1000) was from DAKO, whereas anti-ubiquitin Lys-48 clone Apu2 was from MILLIPORE (Billerica, MA). HRP-conjugated goat anti-mouse IgG (H + L) was from Zymed Laboratories Inc. (1:5000, Carlsbad, CA), whereas the HRP-donkey anti-rabbit (1:5000) was from GE Healthcare (Buckinghamshire, UK). FITC-conjugated rabbit anti-mouse and TRITC-conjugated mouse anti-rabbit antibodies were from DAKO.

Cell Lines—The BJAB-t-tAEBNA1 cell line was described elsewhere (19). The BJAB-t-tAEBNA1AGA cell line was generated by transfection of the pTRE2pur-EBNA1ΔGA plasmid into BJAB-t-tA using the Nucleofector® kit V, program G10 (Lonza, Basel, Switzerland), followed by selection in RPMI 1640 complete medium containing 1 μg/ml puromycin and 500 μg/ml hygromycin B. Semiconfluent monolayers of HeLa cells were transfected in 60-mm dishes with 0.5 μg of the indicated plasmid using the JetPei transfection kit (Polyplus Transfection, Illkirch, France).

Plasmids—The pTRE2pur-EBNA1ΔGA plasmid was described elsewhere (19). pTRE2pur-EBNA1ΔGA was generated by swapping a BspEI/NotI fragment from pFLAG-EBNA1ΔGA into pTRE2pur-EBNA1. EBNA1ΔGA-NLS was generated by overlapping PCR, using the primers 5′-CAAGCTTGTGACCATTGACTACTAAAGGAGCC-3′ to 5′-TATGGACTACAAGGACGACGATGACAA-3′. The fragments were cloned in the HindIII/NotI restriction sites of p3FLAG-CMV-VK to generate 3FLAG-CMV-VK. The PCR product was cloned in the HindIII and NotI restriction sites of the pcDNA3 (Invitrogen) and pBCKMV (Stratagene, La Jolla, CA) vectors. pCIT-NotI fragments encoding GA25, GA121 (12), and GA239 (11) were cloned in the HindIII/XhoI restriction sites of p3FLAG-CMV-VK to generate 3FLAG-CMV-VK. The PCR product was cloned in the HindIII and NotI restriction sites of the pcDNA3 (Invitrogen) and pBCKMV (Stratagene, La Jolla, CA) vectors. pCIT-NotI fragments encoding GA25, GA121 (12), and GA239 (11) were cloned in the HindIII/XhoI restriction sites of p3FLAG-CMV-VK to generate 3FLAG-CMV-VK. The PCR product was cloned in the HindIII and NotI restriction sites of the pcDNA3 (Invitrogen) and pBCKMV (Stratagene, La Jolla, CA) vectors. pCIT-NotI fragments encoding GA25, GA121 (12), and GA239 (11) were cloned in the HindIII/XhoI restriction sites of p3FLAG-CMV-VK to generate 3FLAG-CMV-VK. The PCR product was cloned in the HindIII and NotI restriction sites of the pcDNA3 (Invitrogen) and pBCKMV (Stratagene, La Jolla, CA) vectors. pCIT-NotI fragments encoding GA25, GA121 (12), and GA239 (11) were cloned in the HindIII/XhoI restriction sites of p3FLAG-CMV-VK to generate 3FLAG-CMV-VK. The PCR product was cloned in the HindIII and NotI restriction sites of the pcDNA3 (Invitrogen) and pBCKMV (Stratagene, La Jolla, CA) vectors.
CTCAGTAAGCTTTTTT-3’ and 5’-AAAAAGTTTACCC-TAAGAGACCTCGGGGCACAAAGGACTCGAG-AAA-3’ and 5’-TTTCTGAGTCCCTTGTGCGGGCCCAGGTTCTTCATTGTTAGTGAAGC-3’, respectively. AT1/2-VK and AT1/3-VK were generated by mutagenesis using the primer pairs 5’-AGCGAAGTGGCAAGGCAAAGAAACAG3’ and 5’-CTGTGGTTTGGCTGGACATTGCCTGCT-3’ and 5’-AGAAAAAACAGCTCGAGAATT-3’ and 5’-AATT-TTCTCAGCTTTTGTGGT-3’.

Immunoprecipitation—Semiconfluent monolayers of HeLa cells grown in 60-mm Petri dishes were transfected with 1 μg of the indicated plasmid. After 24 h, each plate was split into two and cultured for an additional 24 h. One plate of each duplicate was treated with 10 μM MG132 for 6 h before lysis. To break non-covalent protein interactions, the cells were lysed for 30 min at room temperature in 100 μl of denaturing buffer containing: 1% SDS, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM iodoacetamide, 10 mM N-ethylmaleimide, and protease inhibitors. The lysates were then diluted 10 times with a non-denaturing buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 20 mM iodoacetamide, 10 mM N-ethylmaleimide, and protease inhibitors). Protein concentration was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). The lysates were fractionated in NUPAGE Bis-Tris precast gels (Invitrogen) and blotted on nitrocellulose membranes (Amersham Biosciences Tokyo, Japan) and analyzed with Adobe Photoshop©. Statistical Analysis—Data are shown as mean ± S.E. of three or more experiments. Statistical analysis was performed using Student’s t test, two-way analysis of variance, and Pearson correlation coefficient. p values ≤0.05 were considered as significant.

RESULTS

The GAr Stabilizes EBNA1 in the Nucleus—Conditional transfectants of the BJAB-tTA cell line were generated to examine the short- and long-term turnover of EBNA1 and EBNA1-ΔGA. The polypeptides were not detected by Western blots in transfected cells cultured in the presence of 1 μg/ml Dox but were rapidly expressed upon Dox removal, reaching comparable plateau levels with 8–10 days of culture (Fig. 1A). Short-term protein turnover was assessed by CHX chase in cells expressing steady state levels of EBNA1 or EBNA1-ΔGA (Fig. 1B). The intensities of the specific bands detected in Western blots probed with the EBNA1 specific antibody OT-1x were virtually unchanged during 9 h of chase, indicating that both EBNA1 and EBNA1-ΔGA are long-lived proteins.

CHX treatment may affect the turnover of proteasomal substrates by altering the expression of short-lived components of the proteolytic machinery. To address this possible artifact, we took advantage of the regulated expression of EBNA1 and EBNA1-ΔGA that allows rapid shut off of transcription by the addition of Dox and long-term monitoring of protein turnover under physiologic conditions. Representative Western blots illustrating protein decay over a period of 72 h are shown in Fig. 1C, and the relative rate of decay expressed as EBNA1-ΔGA/EBNA1 ratio in three independent experiments is summarized in Fig. 1D. Taking into account the dilution effect of cell proliferation, both EBNA1 and EBNA1-ΔGA appeared to be long-lived. However, although most of the input EBNA1 was recovered after 72 h, EBNA1-ΔGA exhibited a faster turnover with 50% decrease relative to EBNA1 in ~24 h and 90% decrease within 72 h (p = 0.0028). Thus, although the GAr appears to be required for full stabilization, other properties of EBNA1 participate in the regulation of protein turnover.

Because EBNA1 is a nuclear protein and binds to cellular DNA (22), we tested whether these features may account for the inefficient degradation of EBNA1-ΔGA. The nuclear import of EBNA1 is dependent on the presence of a nuclear localization signal (NLS) located between amino acids 379 and 387 of the prototype B95.8 encoded protein and on interaction with importin-α (23). To assess the effect of nuclear localization on EBNA1 stability, an E1-ΔGA-NLS mutant was generated by deleting the NLS from EBNA1-ΔGA. Staining of transiently transfected HeLa cells with the EBNA1 specific antibody K67.3 confirmed that E1-ΔGA-NLS accumulates in the cytoplasm (Fig. 2A). Representative Western blots illustrating the decay of EBNA1, EBNA1-ΔGA, and EBNA1-ΔGA-NLS in a 9-h CHX

1 h with the appropriate FITC- or TRITC-conjugated secondary antibodies, the slides were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA). Digital images were captured using a LEITZ-DMRB fluorescence microscope (Leica, Wetzlar, Germany) equipped with a CCD camera (Hamamatsu Photonics KK, Tokyo, Japan) and analyzed with Adobe Photoshop©.

Protein Stabilization by High Avidity DNA Binding

Assays of Protein Turnover—Short-term protein turnover was assayed by chase in medium containing 60 μg/ml CHX. The cells were lysed in radioimmune precipitation buffer (150 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1% Triton, 0.1% SDS, and protease inhibitors), and protein concentration was measured using the Bio-Rad DC protein assay kit (Bio-Rad Laboratories). The lysates were fractionated in NUPAGE Novex® Bis-Tris precast gels (Invitrogen) and blotted on 0.2-μm nitrocellulose membranes (Amersham Biosciences Hybrid ECL, GE Healthcare). After staining with the indicated primary and secondary antibodies, immunocomplexes were detected by chemiluminescence (PIERCE®ECL, Thermo Scientific). Long-term protein turnover was assayed in conditional transfecteds after the addition of 1 μg/ml doxycycline. The dilution effect of cell proliferation was accounted for by monitoring the number of live cells by trypan blue dye exclusion.

Immunofluorescence Staining—HeLa cells grown on round cover slides were transfected with 0.5 μg of the indicated plasmid using the JetPej kit. After 48 h, the slides were fixed with 4% formaldehyde in PBS for 20 min followed by permeabilization and blocking for 30 min in a solution containing 1% Triton X-100 and 3% BSA in PBS. Where indicated, detergent extraction was performed before fixation by incubating the slides for 5 min on ice in buffer containing 137 mM NaCl, 20 mM d-glucose, 20 mM HEPES, 5.4 mM KCl, 1.8 mM CaCl2, 0.8 mM MgSO4, and 0.5% Triton X-100, pH 7. After staining at room temperature for 1 h with anti-FLAG or anti-EBNA1 antibodies and for 1 h with rhodamine-conjugated secondary antibody, the slides were mounted with VECTASHIELD mounting medium containing DAPI (Vector Laboratories Inc., Burlingame, CA). Digital images were captured using a LEITZ-DMRB fluorescence microscope (Leica, Wetzlar, Germany) equipped with a CCD camera (Hamamatsu Photonics KK, Tokyo, Japan) and analyzed with Adobe Photoshop©.

Statistical Analysis—Data are shown as mean ± S.E. of three or more experiments. Statistical analysis was performed using Student’s t test, two-way analysis of variance, and Pearson correlation coefficient. p values ≤0.05 were considered as significant.
Protein Stabilization by High Avidity DNA Binding

FIGURE 1. Both EBNA1 and EBNA1-ΔGA are long-lived proteins. The EBNA1 and EBNA-ΔGA coding regions were cloned in the pTRE2pur vector under control of a tetracycline-regulated promoter and stably transfected in the BJAB-tTA cell line. A, one representative Western blot where total cell lysates of cells cultured in the presence or absence of 1 μg/ml Dox were probed with the EBNA1 specific antibody K67.3. GR1 = GRr subdomain 1 (amino acids 54–89); GR2 = GRr subdomain 2 (amino acids 331–361); NLS = nuclear localization signal (amino acids 379–386); GAr = Gly-Ala repeat (amino acids 89–328); DBD = DNA binding domain (amino acids 451–651). B, the short-term turnover of EBNA1 and EBNA1-ΔGA was assessed by chase for 9 h in medium containing 60 μg/ml CHX. One representative Western blot out of three is shown in the figure. C, the long-term turnover of EBNA1 and EBNA1-ΔGA was monitored for the indicated times after the addition of Dox. One representative experiment illustrating the relative decay of the polypeptides detected in Western blots is shown. D, the intensity of the EBNA1 and EBNA1-ΔGA specific bands was quantified by densitometry, and the relative turnover was calculated as EBNA1-ΔGA/EBNA1 ratio. Mean ± S.E. of three experiments (p = 0.0028). The BJAB-tTA-E1 and BJAB-tTA-E1ΔGA cell lines showed a similar replication time of ~24 h.

FIGURE 2. EBNA1-ΔGA is stable in the nucleus but is degraded in the cytoplasm by the proteasome. A, EBNA1-ΔGA/NLS accumulates in the cytoplasm. Transfected HeLa cells were stained with the EBNA1 specific antibody K67.3. B, EBNA1-ΔGA/NLS is a short-lived protein. A representative Western blot illustrating the turnover of EBNA1, EBNA1-ΔGA, and EBNA1-ΔGA/NLS in HeLa cells chased for the indicated time in medium containing 60 μg/ml CHX is shown. C, the intensity of the specific bands was measured by densitometry, and the percentage residual protein was calculated as (intensityt/intensity0) × 100. D, EBNA1-ΔGA/NLS is degraded by the proteasome. Transfected HeLa cells were chased for 9 h in the presence of CHX with or without the addition of 10 μM MG132. Two independent experiments (Exp I and Exp II) are shown.

Thus, although confirming the capacity of the GAr to inhibit proteasomal degradation and supporting our earlier conclusion that EBNA1-ΔGA is a competent proteasomal substrate, these findings identify subcellular localization as an important determinant of EBNA1 turnover.

EBNA1 interacts to cellular DNA through a bipartite GRr domain that flanks the GAr on both sides and anchors the protein to mitotic chromosomes, which is required for correct partitioning of the viral episomes in replicating cells (24). The two chromosome binding subdomains of the GRr were shown to be AT-hooks (25), a DNA binding motif found in the architectural transcription factors of the high mobility group A (HMGa) family (25–27). To test whether binding to DNA affects protein stability, an EBNA1-ΔGA/GR polypeptide was expressed in HeLa cells. The subcellular localization of the mutant polypeptide is shown in Fig. 4A, a representative Western blot illustrating protein decay in a 9-h CHX chase is shown in Fig. 4B, and
the amount of residual protein calculated from the densitometry of three independent experiments is shown in Fig. 4 C. In line with the continued presence of the NLS, EBNA1-ΔG/GR retained a predominant nuclear localization. Deletion of the GRr was associated with accelerated protein turnover corresponding to a half-life of ~4 h. Protein degradation was inhibited by the addition of MG132 (Fig. 4 D), supporting the conclusion that in the absence of the GAR, binding to DNA via the GRr may be sufficient to protect EBNA1 from proteasomal degradation.

**The GRr Is a Portable Inhibitor of Proteasomal Degradation**

To further explore the effect of the GRr, an EBNA1 fragment encompassing the two subdomains located on either side of the GAR (Fig. 1 A) was fused to an immunoglobulin VK polypeptide that is efficiently degraded by the proteasome (Fig. 5 A) and, due to the small size, can freely diffuse in and out of the nucleus. FLAG-VK chimeras containing the GAR (VK-GAR), the nuclear localization signal of EBNA1 (NLS-VK), the bipartite GRr domain (GR1/2-VK), or both the NLS and the GRr (GR1/2-NLS-VK) (Fig. 5 B) were compared for their subcellular localization and rate of turnover in a 6-h CHX chase. In accordance with the small size, VK and VK-GAR were evenly distributed throughout the cell, and insertion of the NLS induced nuclear accumulation, as expected (Fig. 5 C). The GR1/2-VK chimera showed exclusive nuclear localization even in the absence of the NLS (Fig. 5 C), indicating that the GRr is sufficient to tether the protein to cellular DNA, which was further supported by the virtually exclusive association of the chimera to mitotic chromosomes (Fig. 5, D and E). Insertion of the NLS alone did not affect the turnover of VK, which remained well below 2 h, whereas insertion of the GAR stabilized VK as efficiently as insertion of the GRr (Fig. 5, F and G). Thus, the GAR and GRr serve as independent cis-acting stabilization signals that inhibit proteasomal degradation.

**High Avidity Binding to DNA Prevents the Degradation of Ubiquitylated Substrates**

The GRr binds to cellular DNA via AT-hook-like motifs that interact with AT-rich regions in the minor groove of DNA (27). To obtain independent evidence for the effect of DNA binding on protein stabilization, we compared the effect of a set of known chromatin binding domains. To this end, FLAG-VK was fused to the AT-hook of HMGA-1a (AT1/2/3), the A/B box of HMGB-1 (A/B box), and histone H1, which interact with partially overlapping sites in the minor groove (28), and with the DNA-targeting module of histone-lysine N-methyl-transferase SUV4-20h2 (SUV-DBD), which interacts with pericentric heterochromatin (29) (Fig. 6 A).
Although HMGA and HMGB proteins are highly mobile in the nucleus, as determined in fluorescence recovery after photobleaching assays (30), histone H1 and SUV-4-20h2 are virtually immobile (29, 31). As expected, fusion of histone H1, the AT-hook of HMGAa1, and SUV-DBD at the N terminus of VK induced exclusive nuclear localization, whereas A/B box-VK showed some residual cytoplasmic fluorescence (Fig. 6, left panels). To further discriminate between different types of DNA binding, fluorescence assays were also performed on cells treated with 0.5% Triton X-100 prior to fixation, which extracts proteins loosely bound to chromatin (32). This treatment reduced significantly the nuclear fluorescence of A/B box-VK and SUV-DBD-VK, whereas the fluorescence of GR1/2-VK, AT1/2/3-VK, and H1-VK remained unchanged (Fig. 6, B, right panels, and C), suggesting higher binding avidity. When protein turnover was assayed by CHX chase, the GR1/2, AT1/2/3, and histone H1 protected VK from proteasomal degradation, whereas the SUV-DBD and A/B box had negligible effects (Fig. 6, D and E). A strong positive correlation ($r = 0.94654$) was observed by plotting the amount of residual fluorescence following Triton X-100 extraction versus the amount of residual protein after a 6-h CHX chase (Fig. 6F), suggesting that the capacity of the domains to inhibit proteasomal degradation is directly proportional to their capacity to induce high avidity binding to DNA.

To explore this possibility further, we took advantage of the previous finding that the architecture of the GRr and AT-hook, fashioning multiple properly spaced DNA binding motifs, is required for high avidity interaction with DNA (30) and constructed FLAG-VK chimeras fused to either the GR1 or the GR2 alone (Fig. 7) or to different combinations of the AT1, AT2, and AT3 subdomains of the AT-hook (Fig. 8). Neither the GR1 nor the GR2 subdomain conferred Triton X-100-resistant binding to DNA, although the GR2 domain induced virtually
exclusive nuclear localization (Fig. 7B). In accordance, neither domain alone protected VK from proteasomal degradation (Fig. 7, C and D). As illustrated by the representative fluorescence and Western blot assays shown in Fig. 8, B and C and summarized in Fig. 8D, comparable results were obtained with chimeras containing different combinations of the AT1, AT2, and AT3 motifs. Only the intact AT1/2/3 domain induced detergent-resistant binding to DNA and protected VK from proteasomal degradation.

Binding to DNA may sterically interfere with substrate ubiquitylation or may influence the efficiency of ubiquitylation. To address these possibilities, accumulation of ubiquitylated VK, AT1/2/3-VK, and GR1/2-VK was induced by treating transfected HeLa cells with the proteasome inhibitor MG132. The polypeptides were then immunoprecipitated from cell lysates prepared in the presence of 1% SDS, to destroy non-covalent interactions, and the cysteine protease inhibitor N-ethylmaleimide, to inhibit the activity of ubiquitin deconjugases. Sequential probing of Western blots with the anti-FLAG (Fig. 9A, upper panel) and anti-ubiquitin antibodies (Fig. 9A, middle panel) demonstrated comparable levels of high molecular weight species corresponding to ubiquitylated polypeptides. Reprobing the blots with anti-Lys-48-linked ubiquitin chain antibodies did not reveal consistent changes in the pattern of ubiquitylation (Fig. 9A, lower panel), although the results obtained with this antibody were more variable, probably reflecting a weaker binding to the immunocomplexes. Furthermore, although Triton X-100 extraction before lysis resulted in loss of both unmodified and ubiquitylated VK, the amount of unmodified and high molecular weight AT1/2/3-VK and GR1/
2-VK detected by the anti-FLAG antibody remained unchanged (Fig. 9B), confirming that the ubiquitylated substrates remain firmly bound to DNA.

**DISCUSSION**

Proteasomal degradation is a complex process whose molecular details and multifaceted regulation are only partially understood. Sequences in the substrate that interfere with the process are powerful tools toward the elucidation of critical events leading to proteolysis. Using as a model the EBV nuclear antigen EBNA1, we have identified high avidity binding to DNA, as exemplified by resistance to detergent extraction, as a critical feature that halts the degradation of ubiquitylated substrates by the proteasome.

The bipartite GRr domain of EBNA1 resembles the AT-hook of HMGA proteins that bind in a sequence-independent manner to AT-rich stretches of DNA via three conserved Pro-Arg-Gly-Arg-Pro motifs. The structural and functional similarity between the two DNA binding domains is confirmed by the finding that the AT-hook of HMGA1 can substitute for the GRr in all the functions of EBNA1 that require interaction with cellular DNA, including partitioning of the viral episomes in latently infected proliferating cells (33). NMR studies indicate that the AT-hook undergoes a structural transition upon DNA binding, assuming a crescent-shaped conformation that fits deep into the narrow minor groove (26). Hydrophobic interactions of the arginine side chains with adenines place the domain in a fixed orientation toward DNA (34), whereas the coopera-

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**FIGURE 7.** The bipartite GRr is required for avidity binding to DNA and inhibition of proteasomal degradation. A, schematic representation of FLAG-VK polypeptides fused to the bipartite GRr domain (GR1/2-VK) or the GR1 (GR1-VK) or GR2 (GR2-VK) subdomains. B, subcellular localization of the FLAG-VK chimeras detected by anti-FLAG immunofluorescence. In the left panels, the slides were preincubated for 5 min in buffer containing 0.5% Triton X-100 before fixation. C, representative Western blot illustrating the turnover of the chimeras in a 6-h CHX chase. Insertion of the entire GRr domain is required for inhibition of protein turnover. D, the intensity of the specific bands was quantified by densitometry, and the percentage of residual protein was calculated as (intensity t₀/intensity tₙ) × 100. The means ± S.E. of three independent experiments are shown in the figure.

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**FIGURE 8.** The intact AT-hook is required for high avidity binding to DNA and inhibition of proteasomal degradation. A, schematic representation of FLAG-VK chimera fused to different combinations of the three subdomains of the AT-hook of the HMGA-1A. B, representative micrographs illustrating the subcellular localization of the chimeras and sensitivity to detergent extraction. Fusion to the tripartite AT-hook is required for nuclear sequestration of the VK and detergent-resistant binding to DNA. C, representative Western blots illustrating the turnover of the AT-chimeras in a 6-h CHX chase. D, summary of two independent experiments where sensitivity to detergent extraction and protein turnover were tested in parallel.
The functional significance of this type of DNA interaction is supported by the finding that, like the AT-hook, histone H1 can substitute for the GRr in mediating the association of EBNA1 with mitotic chromosomes, and this correlates with the capacity of EBNA1 to promote the accumulation of plasmid containing the origin of latent viral replication, oriP, and with the long-term persistence of EBV episomes in virus-infected cells (36).

Stabilization of proteasomal substrates by binding to DNA was reported in the context of transcription factors such as p53 (37) and MyoD (38) that interact with specific sequences in the major groove of DNA via large structural motifs, such as the helix-loop-helix and zinc finger domains. The stabilizing effect of these DNA binding domains has been attributed to the induction of conformational changes that promote substrate folding and oligomerization, which may hide critical recognition sites for attack by the proteolytic machinery. This was confirmed in the case of MyoD, where DNA binding was shown to inhibit ubiquitylation (38). The DNA binding domains investigated in our work act in a different manner because the avidity of binding rather than conformational effects appears to play critical roles for substrate stabilization. Tethering to DNA via the GRr or the AT-hook did not interfere with the efficiency of ubiquitylation of the VK chimeras that still occurred prevalently, if not exclusively, through Lys-48-linked chains. Furthermore, the majority, if not all, of the ubiquitylated substrates were resistant to detergent extraction, suggesting that they remain bound to DNA. Thus, DNA binding did not affect the
accessibility of the substrate to the ubiquitin conjugation machinery, although we cannot formally exclude that the altered subcellular localization might lead to the recruitment of different E3 ligases. In addition, although the GR2 subdomain of the GRr and different combinations of at least two AT-hook motifs were sufficient to trap the VK polypeptide on DNA, the entire bipartite GRr and tripartite AT-hook were required for both detergent-resistant binding and inhibition of proteasomal degradation, further supporting the role of binding avidity as a critical determinant of protein stabilization.

One interesting possibility suggested by our findings is that stable interaction with DNA may hamper the capacity of the proteasome to pull the ubiquitylated substrate away from the complex. This possibility is in line with observations made by Jung and Lippard (32) in a study aiming to assess the stability and polyubiquitylation of RNA polymerase II stalled at the site of cisplatin-induced DNA damage. Although the chromatin-bound-Rbp1 subunit of the stalled polymerase was efficiently ubiquitylated through different types of Lys linkages both in vitro and in cisplatin-treated HeLa cells, ubiquitylated Rbp1 that remained firmly bound to DNA after detergent extraction was protected from proteasomal degradation, whereas a loosely bound fraction was rapidly degraded, as confirmed by the selective accumulation in the presence of MG132. It is noteworthy that although the recruitment of the proteasome has been taken to suggest that ubiquitylated DNA-bound proteins are degraded in situ while bound to DNA, this has not been formally proven in most cases, and the requirement for an initial DNA dissociation step, mediated by the proteasome itself or by other chaperones such as cdc48/p97 (39), is at least as likely. Given that different DNA events trigger generalized responses that involve the ubiquitylation of many chromatin-bound proteins, the stabilizing effect of binding avidity could provide an effective means to regulate the efficiency of proteolysis in a substrate-specific and possibly time-dependent manner.

The findings that EBNA1 is protected from proteasomal degradation by two stabilization signals, both acting downstream of ubiquitylation, is intriguing. The cis-acting regulation of proteasomal processing and translation efficiency in vitro (40) are the only functions assigned to the GRr. This EBNA1 domain is dispensable for the immortalization of EBV-infected B-lymphocytes in vitro (41) but is likely to play an important role in vivo because all EBV isolates examined so far encode GRr domains, albeit of different length (42). In contrast, analysis of EBNA1 deletion mutants lacking the N-terminal domain has shown that binding to cellular DNA via the GRr is essential for B-cell immortalization (22). The sequence-independent binding to cellular DNA afforded by the GRr may also contribute to the broad rearrangement of cellular transcription observed in EBNA1-expressing cells (43, 44), in a way analogous to the pleiotropic transcriptional effect of architectural transcription factors such as HMG proteins (27). The resistance of EBNA1 to proteolysis is likely to play a pivotal role in the establishment of life-long persistent infections in healthy EBV carriers by allowing the maintenance of sustained levels of the protein in a latent reservoir of non-proliferating memory B-lymphocytes where transcription of the viral genes is tightly down-regulated (45). It remains to be seen how the two domains, which are clearly capable of providing independent stabilization signals upon grafting to unrelated proteasomal substrates, cooperate in regulating the stability of EBNA1 in different cellular compartments.

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