Gly-Ala Repeats Induce Position- and Substrate-specific Regulation of 26 S Proteasome-dependent Partial Processing*[^1]

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Partial degradation or regulated ubiquitin proteasome-dependent processing by the 26 S proteasome has been demonstrated, but the underlying molecular mechanisms and the prevalence of this phenomenon remain obscure. Here we show that the Gly-Ala repeat (GAr) sequence of EBNA1 affects processing of substrates via the ubiquitin-dependent degradation pathway in a substrate- and position-specific fashion. GAr-mediated increase in stability of proteins targeted for degradation via the 26 S proteasome was associated with a fraction of the substrates being partially processed and the release of the free GAr. The GAr did not cause a problem for the proteolytic activity of the proteasome, and its fusion to the N terminus of p53 resulted in an increase in the rate of degradation of the entire chimera. Interestingly the GAr had little effect on the stability of EBNA1 protein itself, and targeting EBNA1 for 26 S proteasome-dependent degradation led to its complete degradation. Taken together, our data suggest a model in which the GAr prevents degradation or promotes endoproteolytic processing of substrates targeted for the 26 S proteasome by interfering with the initiation step of substrate unfolding. These results will help to further understand the underlying mechanisms for partial proteasome-dependent degradation.

Polyubiquitinated substrates are targeted for 26 S-dependent proteasomal degradation by their ubiquitin moiety that is recognized and bound to one of the 19 S regulatory complexes. Prior to degradation, the substrate has to be unfolded by an ATP-dependent mechanism in the 19 S cap structure. This allows the polypeptide to be translocated and threaded into the central chamber of the 20 S complex where three different types of proteolytic activity catalyze the cleavage of peptide bonds, leading to the complete breakdown of the substrate into small peptide fragments (1, 2).

In addition to this classic model of 26 S proteasome-mediated degradation, the proteasome can also partially degrade specific substrates leading instead to the release of larger degradation products with distinct cell biological activity, the so-called regulated ubiquitin proteasome-dependent processing (3–6). For example, the p50 subunit of NF-κB is generated by the processing of the larger p105 protein (7, 8), and it has been suggested that a glycine-rich region in p105 is responsible and necessary for this partial degradation (9, 10). Partial processing also applies to other transcription factors like the yeast NF-κB homologs Spt23 and Mga2 and the Drosophila Ci (3, 4, 11). The direct targeting of proteins carrying polyglutamine repeat sequences, which are characteristic for several neurodegenerative diseases including Huntington disease and spinocerebellar ataxias, for proteasomal degradation also results in partial degradation in vitro and in vivo (12). These different examples highlight the fact that regulated ubiquitin proteasome-dependent processing is an important physiological aspect of the proteasomal activity that might also play a role in the development of different diseases.

Mechanistically partial processing can be explained by two alternative models. The first one proposes that a substrate is threaded into the proteasome by one end and processed until a tightly folded structure disrupts further degradation (the end-first model). The second model suggests that a loop structure is formed at an internal protein site that is then threaded into the proteolytic chamber and cleaved, leading to one part of the protein being degraded and the other being released (the endopeptidase or loop model) (13). This model has been elegantly demonstrated by using circular polypeptides (14) or natural proteins, such as the NF-κB p105 polypeptide (15). The main difference between these two models is whether processing ends, or starts, at the site of the released fragment and, thus, whether the effect is on the elongation or the initiation of substrate processing.

The function of the Gly-Ala repeat (GAr) sequence of the EBNA1 protein of Epstein-Barr virus is to prevent endogenous presentation of EBNA1-derived antigenic peptides for the major histocompatibility complex class I pathway (16). This function of the GAr has been linked to its capacity to prevent its own synthesis (17) and proteasomal degradation (18). It was initially suggested that the GAr interferes with the proteasome-

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[^4]: The abbreviations used are: GAr, Gly-Ala repeat; HA, hemagglutinin; TNF, tumor necrosis factor; E1, ubiquitin-activating enzyme; E3, ubiquitin-protein isopeptide ligase; Ub, ubiquitin.
dependent degradation pathway and abrogates or severely inhibits EBNA1, or any other protein to which it is fused, from being degraded by the proteasome by a yet unknown mechanism downstream of the polyubiquitination process (18–22). More recent reports have linked the GAr with partial proteasome-dependent degradation when fused to the ornithine decarboxylase substrates (23, 24). However, partial degradation of EBNA1 would impair its function, and this is not observed in Epstein-Barr virus-infected cells, indicating that this model cannot fully explain the function of the GAr. This prompted us to look further into the mechanism by which the GAr affects the proteasomal degradation process.

We studied the effects of GAr on proteasome-dependent degradation when fused to the p53 and IκBa reporter proteins that are well known substrates for the ubiquitin-proteasome pathway as well as in its native EBNA1 context. In vivo results showed that GAr does not always act as an inhibitor of degradation, and it can either promote or prevent 26 S-dependent degradation of the same substrate, depending on where in the protein it is located. In all cases where the GAr was associated with preventing degradation, we observed a majority of the substrate being released intact from the 26 S proteasome and at the same time a fraction of the chimeras being partially degraded. These results cannot be explained by previous models in which the GAr would cause a substrate- and position-independent prevention of degradation or would inhibit the processing of the substrate through the 20 S chamber. We instead propose a model in which the GAr is disrupting the unfolding of the substrate. This model not only offers an explanation to the results presented here but also encompasses previous work on how the GAr affects proteasomal degradation and might help to shed light on the mechanisms that control regulated ubiquitin-dependent partial processing.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The GAr-carrying plasmid encoding the full-length 235-amino acid GAr and carrying an HA tag in the N terminus, EBNA1, and EBNAΔGAr have been described previously (17). Each one of the constructs used was made in the following way using the pCDNA3.1 vector. For the GAr-p53 construct, p53 was amplified using the sense primer 5′-GCGCGAATTCTTTAGGAGGCAGTGAGATC-3′ and the antisense primer primer 5′-GCGCTCTAGTGACAGCTGTTGCGCCTTTC-3′ and cloned into the GAr plasmid using EcoRI and XbaI sites. For the p53-GAr construct, p53 was amplified using the sense primer 5′-GCGCGAATTCTTTAGGAGGCAGTGAGATC-3′ and the antisense primer primer 5′-GCGCTCTAGTGACAGCTGTTGCGCCTTTC-3′ and cloned into the GAr plasmid using HindIII and BspEI restriction sites, and then it was cloned in the p53-GAr plasmid in the place of p53. For the HA-p53-GAr construct,IkBa was amplified using the sense primer 5′-GCGCGAATTCTTTAGGAGGCAGTGAGATC-3′ and the antisense primer primer 5′-GCGCTCTAGTGACAGCTGTTGCGCCTTTC-3′ and cloned into the GAr plasmid using EcoRI and XbaI restriction sites, and then it was cloned in the GAr-p53 plasmid in the place of p53. For the IkBa-GAr construct, IkBa was amplified using the sense primer 5′-GCGCGAATTCTTTAGGAGGCAGTGAGATC-3′ and the antisense primer primer 5′-GCGCTCTAGTGACAGCTGTTGCGCCTTTC-3′ and cloned into the GAr plasmid using EcoRI and BspEI restriction sites, and then it was cloned in the GAr-p53 plasmid in the place of p53. For the GAr-HA, the GAr plasmid was digested with HindIII and BspEI restriction sites, and then it was cloned in the GAr-p53 plasmid in front of GAr. For GAr-HA, the GAr plasmid was digested with HindIII and BspEI restriction sites, and then it was cloned in the GAr-p53 plasmid in front of p53. For GAr-p53-GAr-HA, the GAr plasmid in front of GAr was amplified using the sense primer 5′-GCGCGATATCTCCCTCTTTCAGGCGGCCAGCCGCCC-3′ and antisense primer primer 5′-GCGCTGACTACAGTCAGAAGCTGCTGATTGCC-3′ that introduced HindIII and BspEI restriction sites, and then it was cloned in the GAr-p53 plasmid in front of GAr. For GAr-p53-GAr-HA, the GAr plasmid was digested with HindIII and BspEI restriction sites, and then it was cloned in the GAr-p53 plasmid in front of p53. For GAr-p53-GAr-HA, the GAr plasmid in front of p53 was amplified using the same sense and antisense primers as for the p53-GAr construct, and then it was cloned in the GAr-p53-GAr plasmid in front of p53. For GAr-p53-GAr-HA, the GAr plasmid was digested with HindIII and BspEI restriction sites, and then it was cloned in the GAr-p53-GAr plasmid in front of p53. For GAr-p53-GAr-HA, the GAr plasmid in front of p53 was amplified using the same sense and antisense primers as for the p53-GAr construct, and then it was cloned in the GAr-p53-GAr plasmid in front of p53.
with anti-EBNA1 or anti-p53 monoclonal antibodies and protein G-Sepharose. The beads were washed with phosphate-buffered saline and lysis buffer four times and boiled in SDS loading buffer. Immunoprecipitates were analyzed by SDS-PAGE using 4–12% precast gels (Invitrogen). Data were obtained using phosphorimager analysis.

*Pulse Assay*—Transfected H1299 cells were pulse-labeled with \[^{35}S\]methionine (90 μCi of EasyTag Express[^{35}S][^{35}S] Protein Labeling Mix (PerkinElmer Life Sciences)) after being cultured in methionine-free medium and 25 μM proteasome inhibitor MG132 (Merck Biosciences). Then cells were harvested at the indicated time points. Proteins were immunoprecipitated and separated by SDS-PAGE as described above.

**Quantitative Reverse Transcription-PCR**—Total cellular RNA was extracted using TRIzol reagent (Invitrogen). cDNA synthesis was carried out using the Moloney murine leukemia virus reverse transcriptase (Invitrogen). Triplicate samples were subjected to quantitative PCR using LightCycler SYBR Green I and hybridization probe systems (Roche Applied Science). The relative abundance of gene target mRNA was calculated after normalization using the TATA box-binding protein. The primer pairs used for PCR were as follows: p53: forward, 5′-TGGCGCTTTGATGATTGGAAGGAGCTGTGAAA-3′, and reverse, 5′-GCTG-GACTGTCTGTAGATGGC-3′; Bax: forward, 5′-CCTCAAATCGTGCGCCACCT-3′, and reverse, 5′-CATTGTGGGAGGAAGCTGTGAAA-3′; PUMA: forward, 5′-GACCTCAACGCCAAGTA-3′, and reverse, 5′-CTAATTGGGGCTCCATCTT-3′; and Bax: forward, 5′-GCCCTTGTGCTTCAGGGTTT-3′, and reverse, 5′-TCCAATGTCCAGCCCATGAT-3′.

*In Vitro Degradation Assay*—H1299 cells were transfected with p53 or the p53-GAr-HA and GAr-p53-GAr-HA constructs. Cell lysates were then immunoprecipitated with anti-p53 monoclonal antibody and protein G-Sepharose. The beads were washed with phosphate-buffered saline and lysis buffer and then incubated in 26 S proteasome buffer (20 mM Tris-HCl, pH 7.5, 20 mM NaCl, 10 mM MgCl₂, 0.25 mM ATP, 1 mM dithiothreitol) with 400 ng of bacterially expressed hMdm2, 50 ng of E1 (Calbiochem), 50 ng of Ubch5 (Calbiochem), and 1 μg of purified 26 S proteasome (BioMol) at 37 °C for 3 h. The reaction was terminated by adding SDS loading buffer and boiling for 15 min at 85 °C. The results were visualized by Western blot using anti-p53 or anti-GAr polyclonal antibodies.

**Fluorescence Microscopy**—Cells were grown on coverslips. 24 h after transfection cells were washed twice in phosphate-buffered saline and fixed with 20% acetone, 80% methanol for 20 min at −20 °C. The fixative was removed by three phosphate-buffered saline washes, and cells were probed with anti-p53 mouse monoclonal antibody and anti-mouse Texas Red secondary antibody. The nucleus was localized by using 4′,6-diamidino-2-phenylindole staining. Cells were visualized using an LSM 510 META (Carl Zeiss) confocal microscope.

**RESULTS**

*The GAr Does Not Affect EBNA1 Stability*—Earlier studies have suggested that the GAr sequence of EBNA1 protein has the capacity to inhibit ubiquitin-dependent degradation of EBNA1 (18). However, when we compared the rate of degradation of EBNA1 with that of an EBNA1 protein that lacks the GAr (EBNA1∆GAr) in vivo using cycloheximide pulse-chase we found that both proteins have a similar half-life over 8 h (Fig. 1a). It has been suggested previously that the effect of the GAr on EBNA1 turnover rate is not evident after 8 h (18), so to test the half-life of these proteins over a longer time period, cells expressing both constructs were labeled with \[^{35}S\]methionine for 2 h followed by a 12- and 24-h chase in normal medium supplemented with 10 μM methionine. c, H1299 cells were transfected with the Ub-EBNA1 expression construct, and the rate of degradation was analyzed over an 8-h time course in the presence of cycloheximide. Equal loading was confirmed by immunoblotting for actin. All data are representative of at least three independent experiments. *WB,* Western blot. *Error bars* represent S.D.
but that the explanation for this is to be found outside the GAR sequence.

The GAR Affects Proteasomal Degradation in a Substrate- and Position-dependent Way—The capacity of the GAR to mediate protein stability has mainly been studied using substrates other than EBNA1, so we wanted to investigate to what extent the reported effects of GAR on protein degradation are substrate-specific. We constructed a set of chimeras containing the full-length GAR fused to the N or C terminus of p53 (GAR-p53 or p53-GAR, respectively) (Fig. 2a). We chose p53 because it is well known that binding of the Mdm2 E3 ligase to the N terminus of p53 promotes p53 polyubiquitination and its subsequent degradation by the 26 S proteasome (28–30). Hence we could control the degradation of the Gly-Ala-p53 fusion constructs via the ubiquitin-dependent pathway by regulating Mdm2 expression levels.

When we expressed different Gly-Ala-p53 chimeras in H1299 cells (expressing a small amount of endogenous Mdm2) and induced 26 S proteasome-dependent degradation by overexpressing Mdm2, we observed a difference in stability of the fusion constructs depending on the position of GAR. Although fusing the GAR to the C terminus of p53 (p53-GAR) led to protection from Mdm2-mediated proteolysis (Fig. 2b), fusion to the N terminus of p53 (GAR-p53) resulted in a more unstable product compared with p53 itself (Fig. 2c). In the case of GAR-p53, the mRNA translation inhibitory effect of the GAR (17) resulted in a protein in which the rate of degradation exceeded the rate of synthesis in the presence of Mdm2, making pulse-chase quantifications impossible. The effect of the destabilization of the GAR-p53 was only observed in the presence of Mdm2, demonstrating that fusion of the GAR to the N terminus of p53 did not generate an intrinsic unstable protein; this suggests that the GAR-p53 chimera is properly folded and that the observed differences in the stability of the chimeras are 26 S-dependent. This was further supported by the observation that both GAR-p53 and p53-GAR induced the expression of the p53 response genes p21, Bax, and Puma, indicating that these p53 chimeras take an active conformation (supplemental Table 1). There was also no difference observed in the intracellular localization of the GAR chimeras compared with p53 wild type (supplemental Fig. 1a), further confirming that GAR-carrying chimeras are properly folded and do not form aggregates. We
also investigated whether fusion of the GAr to the N or C terminus of p53 affects Mdm2-dependent ubiquitination. Treatment of cells expressing p53 or p53-GAr chimeras with proteosome inhibitors revealed no significant differences in the relative amounts of ubiquitination of the chimeras (supplemental Fig. 1b); this is in line with previous reports that the GAr acts downstream of the polyubiquitination process (19, 22). Hence fusion of the GAr to either end of p53 altered 26 S-dependent turnover rate but did not affect the intrinsic stability, the localization of the fusion protein, or the capacity of Mdm2 to promote polyubiquitination. Importantly the fact that the entire GAr-p53 chimera was degraded by the proteosome shows that GAr does not impose any physical hindrances for the proteasomal catalytic activity nor does it prevent the interaction of substrates with the proteasome as has been suggested previously (19, 31).

To see to what extent the position-dependent effect of GAr on proteosome-mediated proteolysis of IκBα on the N and C termini of IκBα (GAr-IκBα and IκBα-GAr, respectively) (Fig. 2a). Extracellular stimulation by a variety of sources, including TNFα, results in the activation of the IκB kinase complex whereupon IκBα is phosphorylated and rapidly ubiquitinated and targeted for 26 S proteasome-mediated degradation (32). As shown in Fig. 2d, 30-min treatment with TNFα in HeLa cells, in which TNFα-dependent degradation of IκBα is known to be efficient, resulted in a sharp reduction in the expression levels of an HA-tagged IκBα. Quantification of the Western blot showed that under the same conditions the levels of GAr-IκBα were reduced by approximately 40% and the levels of IκBα-GAr were reduced by approximately 15% compared with a 70% reduction in the levels of HA-IκBα wild type. Thus, the effect of GAr on ubiquitin-dependent degradation of IκBα is also position-dependent; however, unlike p53, the GAr does not promote the turnover of IκBα.

To further confirm that the results observed were proteasome-dependent, the p53-GAr and IκBα-GAr transfectants were treated for 2 h with the proteasome inhibitor MG132 (supplemental Fig. 2). It should be noted that the effect of proteasome inhibitors on Mdm2-dependent degradation of GAr-p53 constructs was lesser compared with GAr-IκBα constructs where the inhibitors were added before or at the same time as the degradation signal.

GAr-carrying Chimeras Are Partially Processed by the 26 S Proteasome—The position-dependent effect of GAr on proteasomal degradation has not been observed previously, so we wanted to investigate this further. Interestingly immunoblotting using an anti-GAr-specific polyclonal antibody revealed the existence of a band of approximately 235 amino acids in p53-GAr constructs where the inhibitors were added before or at the same time as the degradation signal.

FIGURE 3. GAr-carrying chimeras are partially processed by the 26 S proteasome. a, H1299 cells expressing p53-GAr were targeted for 26 S-dependent degradation by overexpressing Mdm2. Immunoblotting using an anti-GAr-specific polyclonal serum revealed a band corresponding to the full-length 235-amino acid GAr in cells co-expressing p53-GAr and Mdm2. The F19A-mutated p53 protein cannot be targeted for degradation by Mdm2 (25), and free GAr* was not detected in cells expressing p53F19A-GAr in the presence of Mdm2. The gel was overexposed to show the presence, or absence, of the free GAr product. b, in vitro degradation of p53-GAr using purified 26 S proteasome as described under “Experimental Procedures.” c, fusion of the GAr to the N terminus of p53 (GAr-p53) does not lead to protection from Mdm2-dependent degradation or in the release of the free GAr*. d, HeLa cells expressing the IκBα-GAr fusion protein show an increase in the amount of free GAr* after TNFα treatment that was suppressed in the presence of 20 μM of the proteasome inhibitor MG132. All data are representative of at least three independent experiments. * indicates that the polypeptide is a degradation product. The corresponding less exposed gels are shown in supplemental Fig. 3. wt, wild type; WB, Western blot; IP, immunoprecipitation.
GAr chimera for the 26 S-dependent degradation pathway. This was further confirmed in in vitro experiments where we observed the appearance of a GAr product derived from the p53-GAr in the presence of purified 26 S proteasomes (Fig. 3b).

Notably the GAr band was not visible when GAr was fused to the N terminus of p53 (GAr-p53), showing that this construct is completely degraded when targeted for the proteasome by Mdm2 (Fig. 3c and supplemental Fig. 3b). Expression of the IκBα-GAr fusion protein that was shown to be protected from degradation (Fig. 2d) also resulted in the appearance of the free GAr (Fig. 3d and supplemental Fig. 3c). The amount of free GAr was increased after TNFα treatment and was prevented by the addition of proteasome inhibitor, demonstrating that this effect is 26 S-dependent.

Our results taken together so far demonstrate the following. (i) The GAr can prevent 26 S proteasome-dependent degradation of substrates to which it is fused, and this is linked to a fraction of the substrate being partially processed (e.g. p53-GAr and IκBα-GAr); (ii) the GAr can promote 26 S-dependent degradation and be completely degraded along with the rest of the fusion protein (e.g. GAr-p53); or (iii) the GAr can impose little, or no, effect on protein stability (e.g. EBNA1).

The GAr Is a Specific Regulator of the 26 S Proteasome—Several amino acid repeat sequences have been shown to interfere with the ubiquitin-proteasome pathway. The most commonly known example is the polyglutamine repeats (poly(Q)), associated with Huntington disease (HD) or spinocerebellar ataxias SCA 1–7, which are dominantly inherited neurodegenerative diseases. These diseases are caused by abnormal expansions of long glutamine sequences found normally in certain proteins (e.g. huntingtin or ataxin) (33). The poly(Q) sequences in huntingtin from normal individuals are 6–36 residues long; however, in typical Huntington patients, they exceed 36 residues, and the protein has a strong tendency to form aggregates containing large amounts of ubiquitin, components of the 26 S proteasomes, and molecular chaperones, indicating unsuccessful attempts by the cell to refold or destroy these toxic protein aggregates (34–36).

To test the specificity of the effects of GAr on proteasomal degradation, we fused an 80-amino acid-long poly(Q) to the N or C terminus of p53 and expressed these constructs in H1299 cells. As shown in Fig. 4a, insertion of the poly(Q) sequence in either end of p53 resulted in the stabilization of the chimeras (poly(Q)-p53 or p53-poly(Q)). Unlike the GAr, the stabilization conferred by the poly(Q) repeat was not accompanied by partial degradation of the fusion proteins (Fig. 4b). Similar to the GAr, however, the poly(Q) repeat also led to the accumulation of polyubiquitinated products. Hence both repeat sequences act downstream of the ubiquitination process, but only the GAr seems to cause regulated ubiquitin proteasome-dependent processing in a position- and substrate-dependent fashion, demonstrating the unique features of the GAr as a specific regulator of the 26 S proteasome.

The 26 S Proteasome Degrades the N-terminal Part of GAr-carrying Chimeras—The capacity of GAr to protect the entire chimera from 26 S-dependent degradation and at the same time induce partial degradation of a fraction of the substrates is puzzling and cannot be easily explained by any existing models for proteasomal degradation. So we next set out to investigate this phenomenon in more detail. We first made a triple construct coding for two p53 proteins separated by the GAr carrying an HA tag at its N terminus (HA-p53-GAr-p53). This construct allowed us to test whether GAr is able to exert its role on inhibiting degradation and provoking partial proteolysis when it is encircled by two proteins that consist of good substrates for proteasomal degradation. It also allowed us to test whether the difference in the rate of degradation of GAr-p53 and p53-GAr is because of effects related to an N- or C-terminal initiation of p53 degradation.

When we expressed HA-p53-GAr-p53 in H1299 cells, we detected the full-length triple construct and a band corresponding to the GAr-p53* (indicates a degradation product) that was recognized by both anti-p53 (Fig. 5a, left panel) and anti-GAr antibodies (data not shown). Because this band was not recognized by the anti-HA tag antibody (Fig. 5a, right panel) we can conclude that it corresponds to the GAr-p53* part of the triple construct. The GAr-p53* fragment appeared to be decreased when cells were co-transfected with Mdm2 (Fig. 5a, left panel), suggesting that this fragment is further degraded by the proteasome. To test whether this GAr-p53* is indeed a product of proteasomal degradation, we treated cells with the specific proteasome inhibitor epoxomicin. This resulted in a net increase in the expression levels of HA-p53-
GAr Disrupts 26 S Proteasomal Activity

FIGURE 5. The 26 S proteasome degrades the N-terminal part of GAr-carrying chimeras. a, fusion of p53 to either end of GAr (HA-p53-GAr-p53) results in degradation of the p53 sequence linked to the N terminus of GAr and the release of the free GAr-p53*. Treatment with epoxomicin for 5 h results in increased levels of HA-p53-GAr-p53, whereas GAr-p53* levels are slightly decreased. The graph shows the quantification of the corresponding immunoblot (upper left panel). The levels of the triple chimera do not change when cells are treated with several other protease inhibitors (middle panel). The HA-GAr-p53 polypeptide expressed from the HA-GAr-p53 mRNA was detected using an anti-HA tag antibody, but the GAr-p53* that is derived from degradation targeted for degradation by Mdm2 (Fig. 5a, left panel). b, an autoradiograph showing a 30-min [35S]methionine pulse label of cells expressing HA-GAr-p53 and HA-p53-GAr-p53 in the presence or absence of MG132 followed by an immunoprecipitation (IP) using an anti-HA tag monoclonal antibody. c, HA-IκBα-GAr-p53 fusion protein is partially degraded by the 26 S proteasome. Both the triple HA-IκBα-GAr-p53 and the GAr-p53* product are targeted for degradation by Mdm2 (left panel). When cells are treated with TNFα only the triple construct is targeted for degradation, leading to an accumulation of GAr-p53* (right panel). Treatment with epoxomicin prevents Mdm2- or TNFα-dependent degradation of HA-IκBα-GAr-p53 and results in less GAr-p53*. The graphs show the quantification of the corresponding Western blots (WB), and the HA-IκBα-GAr-p53 in the absence of Mdm2 or TNFα has been given the arbitrary value 100. All data are representative of at least three independent experiments. * indicates that the polypeptide is a degradation product. AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride. Error bars represent S.D.

GAr-p53, whereas the amount of GAr-p53* was slightly decreased (Fig. 5a, left panel) despite the fact that GAr-p53* was also subject to proteasome-dependent degradation. Importantly when cells were treated with several other protease inhibitors we did not observe significant differences in the ratios of HA-p53-GAr-p53 to GAr-p53* levels (Fig. 5a, middle panel). A metabolic pulse labeling using [35S]methionine in the presence or absence of proteasome inhibitors showed that the GAr-p53* was not derived from partial translation of the HA-p53-GAr-p53 message (Fig. 5b).

Similarly we constructed a chimera where GAr is inserted between the IκBα and p53 proteins (HA-IκBα-GAr-p53). This fusion protein could be targeted for 26 S proteasome-dependent degradation both by Mdm2 or TNFα. Immunoblotting using a p53 antibody revealed the presence of bands corre-
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To further investigate the effect of GAr on proteasome-dependent degradation, we constructed p53 and IκBα chimeras containing the full-length 235-amino acid-long GAr in their N or C termini (Fig. 2a). To ensure that the GAr is not causing aggregates under the conditions used here, we performed immunohistochemistry analysis (supplemental Fig. 1a) and tested the activity (supplemental Table 1) and ubiquitination (supplemental Fig. 1b) of the fusion constructs. When we looked at the degradation of p53 and IκBα chimeras in vivo, we found that depending on the substrate were obliged to use a modified in vitro degradation assay where proteins are immunoprecipitated using p53 antibodies and then used for ubiquitination and degradation in the presence of purified 26 S proteasomes, Mdm2, and ubiquitin. The modification of the standard in vitro protocol could explain the relatively inefficient degradation of the p53 control protein observed in vitro. Nevertheless we were still able to confirm that it was ubiquitinated and slightly degraded (Fig. 6c, left panel). Most importantly, in the case of the GAr-p53-GAr-HA construct, we observed the release of GAr products after the addition of 26 S proteasome (Fig. 6c, right panel). Taken together, these observations suggest that the 26 S proteasome initiates proteolysis of GAr-p53-GAr-HA from an internal site next to the C terminus of p53, thus giving rise to the GAr-p53* that is further degraded by the proteasome and the GAr-HA* that is resistant to degradation. Accordingly the effect of the GAr would not be on the elongation process but on the initiation phase of degradation.

DISCUSSION

The GAr sequence of EBNA1 protein is known to have the capacity to protect proteins to which it is fused from proteasome-dependent degradation. It was initially proposed that the effect of the GAr was substrate-unspecific and caused by a direct inhibition of the proteolytic activity of the proteasome (18–20, 22). Thereafter GAr was characterized as a “stabilization signal” for the ubiquitin-proteasome pathway (37), and it was further proposed that it may have therapeutic applications in gene transfer settings for stabilizing specific proteins (22, 38).

However, there have been many conflicting reports concerning the capacity of GAr to act as a stabilization signal, the physiological reason for this effect, and the underlying molecular mechanism. For example, when a 24-amino acid-long GAr was fused near the N terminus of ErbB-2 protein, a member of the epidermal growth factor receptor family, the chimera was shown to be efficiently degraded by the proteasome (39). Interestingly when GAr was further inserted between amino acids 55 and 56 of ErbB-2, the GAr-containing chimera was even more unstable than the wild type protein, suggesting that the previously reported protective effect of GAr is not valid in this case. Furthermore experiments with N end rule and ubiquitin fusion degradation-targeted green fluorescent protein reporters containing GAr have shown that GAr is not always efficient in protecting chimeras from proteasomal degradation (26). Even EBNA1 itself when targeted for the 26 S proteasome by the fusion of ubiquitin appears to be efficiently and quickly degraded (26, 27).

To further investigate the effect of GAr on proteasome-dependent degradation, we constructed p53 and IκBα chimeras that are known to have the tendency to aggregate because GAr has the capacity to protect proteins to which it is fused from proteasome-dependent degradation. The GAr sequence of EBNA1 protein is known to have the capacity to protect proteins to which it is fused from proteasome-dependent degradation. It was initially proposed that the effect of the GAr was substrate-unspecific and caused by a direct inhibition of the proteolytic activity of the proteasome (18–20, 22). Thereafter GAr was characterized as a “stabilization signal” for the ubiquitin-proteasome pathway (37), and it was further proposed that it may have therapeutic applications in gene transfer settings for stabilizing specific proteins (22, 38).

However, there have been many conflicting reports concerning the capacity of GAr to act as a stabilization signal, the physiological reason for this effect, and the underlying molecular mechanism. For example, when a 24-amino acid-long GAr was fused near the N terminus of ErbB-2 protein, a member of the epidermal growth factor receptor family, the chimera was shown to be efficiently degraded by the proteasome (39). Interestingly when GAr was further inserted between amino acids 55 and 56 of ErbB-2, the GAr-containing chimera was even more unstable than the wild type protein, suggesting that the previously reported protective effect of GAr is not valid in this case. Furthermore experiments with N end rule and ubiquitin fusion degradation-targeted green fluorescent protein reporters containing GAr have shown that GAr is not always efficient in protecting chimeras from proteasomal degradation (26). Even EBNA1 itself when targeted for the 26 S proteasome by the fusion of ubiquitin appears to be efficiently and quickly degraded (26, 27).

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and on the position where GAr is inserted, we obtained
different results on protein degradation. Although fusion of the
GAr to the C terminus of p53 or to both ends of IκBα
resulted in stabilization of the chimeras, insertion of the GAr
to the N terminus of p53 strongly destabilized the chimera,
leading to its accelerated degradation in the presence of
Mdm2 (Fig. 2). Thus, contrary to what was initially sug-
gested, these data demonstrate that the effect of the GAr on
protein stability is substrate- and position-dependent. By
fusing another repeat sequence in the N or C terminus of
p53, the 80-amino acid-long poly(Q) repeat that like the GAr
has been suggested to affect proteasome-dependent degra-
dation (12, 40), we were able to show that despite the fact
that both repeats act downstream of ubiquitination they
affect 26 S-dependent degradation via different mechanisms,
highlighting the specificity of the GAr on regulating 26 S
proteasome degradation (Fig. 4, a and b).

What is surprising, considering what has been published
previously on the GAr, is our observation that when GAr was
in its natural context, the EBNA1 protein, it had no major
effect on protecting EBNA1 from degradation compared with
a mutant lacking the GAr (EBNAΔGAr) (Fig. 1, a and b). The
turnover of EBNA1 and EBNAΔGAr has been tested
previously in vivo by Masucci and co-workers (18). By using a
recombinant vaccinia virus system in pulse-chase experi-
ments performed in CV1 cells, the authors observed that
EBNA1ΔGAr has a half-life of ~20 h compared with EBNA1
that is stable over a 20-h chase. This difference in the turn-
over of EBNA1 and EBNA1ΔGAr is bigger than the 20%
difference we observed after a 24-h chase in H1299 cells (Fig.
1b). The reason for this discrepancy is not clear, but it is
possible, in view of the fact that strong overexpression of
EBNA1 can lead to aggregates, that the vaccinia expression
system used previously (18) could result in EBNA1 aggre-
gates that would appear on an immunoblot as a “stabiliza-
tion.” But in either case, it is unlikely that such a limited
effect on protein stability could explain how the GAr can
prevent EBNA1 peptides from being presented to the major
histocompatibility complex class I-restricted pathway.
Instead the results presented here support the idea that the
capacity of the GAr to suppress major histocompatibility
complex class I antigen presentation of EBNA1 is due to its
capacity to prevent EBNA1 mRNA translation (17). The rea-
son why EBNA1 turns out to be the only protein tested for
which the GAr has little effect on stability might suggest that
EBNA1 is simply not targeted for degradation via the 26 S
proteasome. This notion is supported by the fact that EBNA1
has never been shown to be a substrate for the ubiquitin-
proteasome pathway or to interact with any putative E3
ligases, and any attempts to demonstrate ubiquitination of
both EBNA1 and EBNA1ΔGAr have failed (18, 41). In con-
trast, when EBNA1 was artificially targeted for the protea-
some by fusing a Ub to its N terminus, it was efficiently and
completely degraded (Fig. 1c).

The observation that GAr was completely degraded in the
context of the Ub-EBNA1 construct (Fig. 1c) or when fused
to the N terminus of p53 (Fig. 2b) shows that GAr does not
cause any problem for the proteolytic activity of the 26 S
proteasome. This contradicts previous reports suggesting
that GAr can inhibit Ub proteasome-dependent proteolysis in
trans (31). It has also been proposed that GAr may act by
preventing the interaction of the substrate with the 26 S
proteasome (19). This is challenged by our observation that
a fraction of the fusion proteins that were protected from
proteolysis was actually partially degraded by the 26 S protea-
some, leading to the release of GAr-containing fragments
(Fig. 3). Hence the GAr does not affect the targeting of sub-
strates to the 26 S proteasome and instead acts downstream
of the recognition and interaction of the substrate with the
proteasome. More recent reports have shown that a 30-
amino acid GAr when fused to the ornithine decarboxylase
causes partial degradation of the chimera in yeast (23, 24).
The authors proposed a model where the ATPases of the 19
S regulatory particle “slip” over the GAr, thereby hindering
translocation of tightly folded domains of the protein into
the 20 S core particle. Hence this model predicts that GAr
acts during the elongation process of degradation. Although
GAr has been suggested to be unstructured and to act as an
independent domain in chimeric proteins (42), the model
proposed by Hoyt et al. (24) cannot explain our results dem-
strating the release of the full-length 235-amino acid-long
GAr from several double and triple chimeras (Figs. 3, 5, and
6). In particular, the partial degradation of the triple chimera
containing GAr on both ends of p53 (GAr-p53-GAr-HA)
(Fig. 6) cannot be explained by a similar model that claims
that GAr prevents the elongation of the substrate through the
chamber of the 20 S core by acting as a “slippery rope.” If
this was the case, we would not expect degradation of GAr-
p53-GAr-HA to take place nor the release of the GAr
domain. Finally the observation that only a fraction of the
protected substrates was partially degraded is also against
the idea that GAr acts on the elongation process of degrada-
tion because in this case it would be expected that all sub-
strate should be partially degraded.

So what can the explanation be for the puzzling position-
and substrate-dependent effect of GAr on 26 S-dependent
degradation, and what is the link between protection from
degradation and partial processing? The effects of GAr on
causing partial processing and also protecting the entire sub-
strate from degradation are likely linked to each other. It is
recognized that binding of a protein to the proteasome by
means of polyubiquitination is not sufficient to ensure the
degradation of a protein, and it is becoming increasingly
evident that protein degradation includes several steps that
depend on different signals or tags within the protein
sequence (43). The tethering, unfolding, and translocation
steps are mediated by degradation signals (e.g. polyubiquiti-
ation) and unfolding tags (e.g. unstructured domains) that
determine where initiation of degradation of the substrate
will occur. In terms of degradation by the 26 S proteasome,
the 19 S ATPases initiate unfolding of the substrate in close
proximity to the degradation signal (43, 44). This might offer
an explanation for the different effects of the GAr on 26
S-dependent degradation. Fusion of the GAr to the C termi-
inus of p53 suppressed degradation in an Mdm2-dependent
fashion (Fig. 1c). Mdm2 promotes polyubiquitination on p53
C-terminal residues (45), and we speculate that this occurs in close proximity to the p53 unfolding tag, which is in accordance with the model that the unfolding tag is close to the polyubiquitination site. Similarly PEST sequences in the C terminus of IkBα can be used as degradation signals (46, 47). Thus, fusion of the GAr to the C terminus of p53 or IkBα would position it near the degradation signal, and this might interfere with the natural unfolding of the substrate, leading to its protection or partial degradation. Instead fusion to the p53 N terminus could promote the unfolding due to the fact that the N terminus of p53 is known to control the folding of its C terminus (48, 49).

Taken together, our results support a model of the GAr impairing unfolding of substrates by the 19 S ATPases of the regulatory particle when fused next to the unfolding tag of a substrate (Fig. 7). This leads to a complete failure of the proteasome to initiate degradation of the main pool of substrate (protection from degradation) or, alternatively, to the formation of a loop structure in a small amount of protein that is endoproteolytically cleaved by the 20 S core particle, resulting in the release of GAr fragments and degradation of the rest of the substrate. This model predicts that the GAr is affecting the unfolding of the substrate that takes place at the 19 S regulatory subunit before processing by the 20 S core particle can start. Hence it can explain how the GAr (i) has no effect or (ii) prevents degradation and causes partial processing of the same substrate depending on its location.

To date only a few examples, including NF-κB (15), Mga2 and Spt23 (13), YB-1 (50), and Gli2/Gli3 (51), have been reported to be endoproteolytically processed by the proteasome, and in most of the cases studies have been carried out in vitro, and cleavage was shown to be mediated by the 20 S proteasome alone. Moreover it still remains unclear what are the properties a protein domain should have to allow specific recognition, endoproteolytic cleavage, and partial processing. Our findings that GAr caused in vivo 26 S-dependent endoproteolytic processing in a substrate- and position-dependent manner indicate a specific role of GAr on protein unfolding and degradation depending on the protein context. The model we propose could help to explain previous confusing reports regarding the role of GAr on protein stability and also how repeat or other sequences, such as the glycine-rich region sequence in NF-κB (15) or the processing determinant domain in Gli2/Gli3 (51), can work as transferable elements and induce partial degradation. Finally our data may be relevant for a better understanding of how substrates are degraded by the 26 S proteasome and may provide some ideas as to how proteins can be manipulated to be selectively stabilized/destabilized or partially cleaved.

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FIGURE 7. Model for GAr-dependent interference with the 26 S proteasome. Fusion of GAr far from the natural unfolding tag of a protein (e.g. in the N terminus of p53) does not prevent 26 S-dependent degradation and results in the complete degradation of the entire chimera by the 26 S proteasome. When GAr is instead fused next to the unfolding tag it impairs unfolding of the substrate, leading to an inhibition of degradation of the majority of the chimeras. In a small fraction of GAr chimeras, however, a loop structure is formed at the unfolding tag and is threaded into the 20 S chamber and endoproteolytically cleaved, leading to the release of GAr fragments and degradation of the rest of the substrate. This model predicts that the GAr is affecting the unfolding of the substrate that takes place at the 19 S regulatory subunit before processing by the 20 S core particle can start. Hence it can explain how the GAr (i) has no effect or (ii) prevents degradation and causes partial processing of the same substrate depending on its location.
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