Inhibition of ubiquitin/proteasome-dependent proteolysis in *Saccharomyces cerevisiae* by a Gly-Ala repeat

Stijn Heessen[1], Nico P. Dantuma[1,*], Peter Tessarz, Marianne Jellne, Maria G. Masucci[*]

Microbiology and Tumor Biology Center, Karolinska Institutet, Box 280, S-171 77 Stockholm, Sweden

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Abstract The glycine-alanine (GA) repeat of the Epstein–Barr virus nuclear antigen-1 inhibits *in cis* ubiquitin-dependent proteolysis in mammalian cells through a yet unknown mechanism. In the present study we demonstrate that the GA repeat targets an evolutionarily conserved step in proteolysis since it can prevent the degradation of proteasomal substrates in the yeast *Saccharomyces cerevisiae*. Insertion of yeast codon-optimised recombinant GA (rGA) repeats of different length in green fluorescent protein reporters harbouring N-end rule or ubiquitin fusion degradation signals resulted in efficient stabilisation of these substrates. Protection was also achieved in rpn10Δ yeast suggesting that this polyubiquitin binding protein is not required for the rGA effect. The conserved effect of the GA repeat in yeast opens the possibility for the use of genetic screens to unravel its mode of action.

Key words: Degron; Epstein–Barr virus; Green fluorescent protein; N-end rule; Proteasome; Ubiquitin fusion degradation

1. Introduction

Compelling evidence places ubiquitin/proteasome-dependent proteolysis at the heart of a plethora of essential cellular functions. The degradation machinery acts via a two-step process. The substrates are first tagged with a high molecular weight ubiquitin chain that is generated by the sequential action of a ubiquitin activase (E1), ubiquitin conjugases (E2), and ubiquitin ligases (E3) [1]. In addition, the requirement for a ubiquitin elongation factor (E4) has been documented for some substrates [2,3]. The E1 activates the carboxy-terminus of ubiquitin through the formation of a thiolester bond between an active cysteine residue and the C-terminal glycine residue of ubiquitin. Through the combined action of E2s and E3s, the C-terminus of ubiquitin is then transferred to the ε-NH3 group of a lysine residue within the substrate. In the subsequent steps the conjugated ubiquitin becomes itself ubiquitinated at Lys48 or, more rarely, Lys29 residues, which, through successive rounds of ubiquitination, results in the formation of a polyubiquitin chain. The polyubiquitinated substrates are subsequently recruited to a large proteolytic complex, the proteasome, where they are deubiquitinated, unfolded, translocated into the cavity harbouring the proteolytic sites, and processively degraded into small peptide fragments [4].

Identification of the signals that determine the rate of turnover of specific substrates and elucidation of their mode of action are prime goals for understanding the regulation of cellular functions by ubiquitin/proteasome-dependent proteolysis. It is well established that substrates carry degradation signals [5], also known as degrons [6], which directly or indirectly facilitate interaction with the E3s [7]. These degradation signals can be short amino acid motifs, globular domains, misfolded protein structures or even oligosaccharide chains [5,8,9]. While some degradation signals are constitutively active others are positively or negatively regulated through various posttranslational modifications [5,10–12]. Although a multitude of different interactions may be generated through these structures and their modification, it seems unlikely that degradation signals would be the only determinants of protein half-life. We have recently postulated that proteins may also harbour stabilisation signals that block or delay proteasomal degradation [13]. A repetitive sequence present in the Epstein–Barr virus (EBV) nuclear antigen (EBNA)-1 fulfils the criteria for this type of regulatory element [14].

EBNA-1 contains a repetitive domain composed solely of glycine and alanine residues that varies in lengths from 60 to more than 300 amino acids in different virus isolates [15]. The glycine-alanine (GA) repeat blocks proteasomal degradation of EBNA-1 [16], which prevents the elimination of the latent viral reservoir by cytotoxic T cells [17]. Interestingly, the GA repeat can also specifically retard or abrogate the degradation of other proteasomal substrates expressed as artificial fusions proteins [18]. Functional GA repeat chimeras have been made for the inhibitor of NF-κB, IκB-α [19], and the tumour suppressor p53 [20]. Although analogous stabilisation signals have not yet been identified in cellular proteins, a glycine-rich region (GRR) in the NF-κB precursor p105 shares some similarities with the viral repeat in that its presence defines the site of proteasomal processing and protects the product from complete degradation [21]. Intriguingly, two yeast membrane transcription factors that are distant homologues of the NF-κB precursor and similarly subject to pro-

*Corresponding authors. Fax: (46)-8-331399.
E-mail addresses: nico.dantuma@mtc.ki.se (N.P. Dantuma), maria.masucci@mtc.ki.se (M.G. Masucci).

[1] These authors contributed equally to this work.

Abbreviations: EBV, Epstein–Barr virus; EBNA, EBV nuclear antigen; E1, ubiquitin activase; E2, ubiquitin conjugase; E3, ubiquitin ligase; E4, ubiquitin elongation factor; GA repeat, glycine-alanine repeat; GFP, green fluorescent protein; UFD, ubiquitin fusion degradation; GRR, glycine-rich region; rGA, recombinant GA repeat

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teasome-dependent processing contain low complexity sequences located at a position corresponding to the GRR [22].

While the precise mode of action of the GA repeat remains elusive, biochemical and cellular studies have narrowed the range of possible targets. We have shown that GA repeat-containing chimeras are efficiently ubiquitinated but fail to establish stable interactions with the proteasome in vivo [16,19], although they can still interact with S5a, a polyubiquitin binding subunit of the proteasome, in vitro [20]. Furthermore, while the GA repeat has exclusively *cis*-stabilising activity in vivo [19,20], a synthetic GA repeat peptide was able to *trans*-inhibit proteasomal degradation in vitro without affecting ubiquitination and while preserving the interaction of the ubiquitinated substrate with S5a [23]. Together these data suggest that the GA repeat and related motifs may affect a critical step in the recognition of ubiquitinated proteins by the proteasome, perhaps by triggering the release of the substrate prior to degradation or, as in the case of the GRR, after partial processing. Characterisation of this putative substrate recognition step and identification of its molecular components could help explain the effect of the viral repeat and may have important implications for the general mode of action of the postulated cellular stabilisation signals.

In this investigation we have addressed these questions by asking whether the mechanism targeted by the GA repeat is evolutionarily conserved. Using artificial green fluorescent protein (GFP)-based proteasomal substrates containing recombinant yeast codon-optimised GA repeats of different length, we show that the repeat stabilises N-end rule and ubiquitin fusion degradation (UFD) substrates in *Saccharomyces cerevisiae*. The demonstration that the activity of the GA repeat is conserved in yeast now allows a genetic approach to the analysis of its mechanism of action.

2. Materials and methods

2.1. Yeast strains and plasmids

All strains are derivatives of DF5 (*lys2-801, leu2-3, -112, ura3-52, his3-Δ200, trp1-1*) [24]. The ubr1Δ, ubc4/ubc5Δ, ufd2Δ, ufd4Δ, and mcb1Δ strains have been described previously [25–28]. The Ub-M-GFP, Ub-R-GFP and Ub^{G76V}-GFP without and with the EBV GA239 [29] were excised with *Eco*RI and *Not*I and cloned in pYES2 (Invitrogen) under the regulation of the galactose-inducible GAL1 promoter.

2.2. Generation of the recombinant GA repeat

The recombinant GA (rGA) repeat was generated by annealing complementary DNA oligonucleotides encoding the octamer GGA-GAGAG at 75°C for 1 h. The sequence of the sense rGA repeat oligo was 5′-GG G*TG TAC* AGA TCT **GGT GGT GCT GGT GCT GGT GCT** GGA TCC C*TG TAC A*GG G-3′ (*Ssp*BI sites, italic; *Bgl*II site, underline; repeat encoding, bold; *Bam*HI, double underline) (Sigma-Genosys). The annealed oligos were digested with *Ssp*BI. The Ub-M encoding part (including the 5′ *Bgl*II and *Bam*HI sites) was removed from Ub-M-GFP by religating the vector after *Bgl*II/*Bam*HI digestion resulting in GFP(ΔUb-X). The *Ssp*BI-digested double-stranded oligonucleotides were cloned into the unique *Ssp*BI site of GFP(ΔUb-X). By successive excision of the repeat from the vector with *Bgl*II and *Bam*HI followed by insertion in the unique *Bam*HI site the GFP(ΔUb-X)-rGA26 (double insertion), GFP(ΔUb-X)-rGA53, GFP(ΔUb-X)-rGA107 and GFP(ΔUb-X)-rGA215 were generated (see Fig. 3). The GFP-rGA fragments were excised with *Pin*AI and *Not*I and used to replace the GFP in the pYES2 Ub-M-GFP, Ub-R-GFP and Ub^{G76V}-GFP plasmids giving rise to their repeat containing counterparts.

2.3. Flow cytometry

Yeast transformed with pYES2 plasmids encoding various Ub-X-GFP fusions were grown successively in medium with glucose, raffinose and galactose as sole carbohydrate source, and then in galactose until midlog phase. The induced cultures were analysed with a FACSort flow cytometer (Becton-Dickinson). The mean fluorescence intensity of yeast transformed with empty pYES2 was subtracted from mean fluorescence intensities of GFP fusion-expressing yeast.

2.4. Western blot analysis

Expression from the GAL1 promoter was induced as described above. Total protein extracts of cultures in midlog phase were obtained by lysis in 1.85 M NaOH and precipitation in 50% trichloroacetic acid [30]. Protein extracts corresponding to 0.25 OD_{600} units were resolved by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA). The membranes were incubated with a mix of two monoclonal anti-GFP antibodies (Roche), followed by incubation with a horseradish peroxidase-labelled secondary anti-mouse antibody. Immunocomplexes were visualised using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

2.5. Pulse chase analysis

Induced midlog-phase yeast cultures were washed once in medium lacking methionine and metabolically labelled for 10 min in medium containing ^{35}S-labelled methionine (Amersham Pharmacia Biotech). An excess of cold methionine was then added and aliquots corresponding to 3 OD_{600} units were taken at the indicated time points. The yeast was lysed in HEPES lysis buffer (50 mM HEPES, 5 mM EDTA, 1% Triton X-100, 150 mM NaCl), supplemented with 20 mM *N*-ethylmaleimide (Sigma-Aldrich) using acid-washed glass beads (Sigma-Aldrich). Crude extracts were precleared overnight with protein A-Sepharose (CL-4B, Amersham Pharmacia Biotech). After incubation with a rabbit polyclonal anti-GFP serum (Molecular Probes), the protein–antibody complexes were precipitated for 3 h at 4°C with protein A-Sepharose beads and washed extensively in ice-cold lysis buffer. The beads were dissolved in loading buffer and the proteins were denatured by boiling before separation by SDS–PAGE. The gel was dried and exposed to a phospho-imager screen, followed by quantification of the radioactive signal using a phospho-imager (Molecular Dynamics) and the ImageQuant software program (Molecular Dynamics).

3. Results

3.1. Generation of fluorescent reporters for ubiquitin-dependent proteolysis in yeast

We have previously described the production of GFP-based proteasome substrates for functional analysis of the ubiquitin/proteasome system in mammalian cells [31,32]. To investigate the functionality of these fluorescent reporters in *S. cerevisiae*, the stable Ub-M-GFP, the N-end rule substrate Ub-R-GFP and the UFD substrate Ub^{G76V}-GFP were expressed from the GAL1 promoter (Fig. 1A). Flow cytometric analysis of wild type yeast expressing the different ubiquitin–GFP fusions showed high fluorescent intensities of yeast expressing the Ub-M-GFP while the expression of Ub-R-GFP and Ub^{G76V}-GFP resulted in fluorescent intensities close to the background levels of yeast transformed with an empty plasmid (Fig. 1B, left panel).

Degradation of the N-end rule substrates in yeast depends on the RING finger-containing E3 ligase Ubr1 [33], while UFD substrates are recognised and ubiquitinated by the HECT (homologous to E6-AP C-terminus) domain E3 ligase UFD4 [26]. In addition, degradation of UFD substrates but not N-end rule substrates depends on the activity of the E2 ubiquitin conjugases UBC4 or UBC5 [28], and the recently identified E4 ubiquitin elongation factor UFD2 [2]. In order to investigate whether the GFP reporters are targeted through the anticipated pathways, we analysed the fluorescent intensities of the yeast mutants ubr1Δ, ubc4/ubc5Δ, ufd4Δ, and ufd2Δ. As expected, the Ub-R-GFP reporter was stabilised

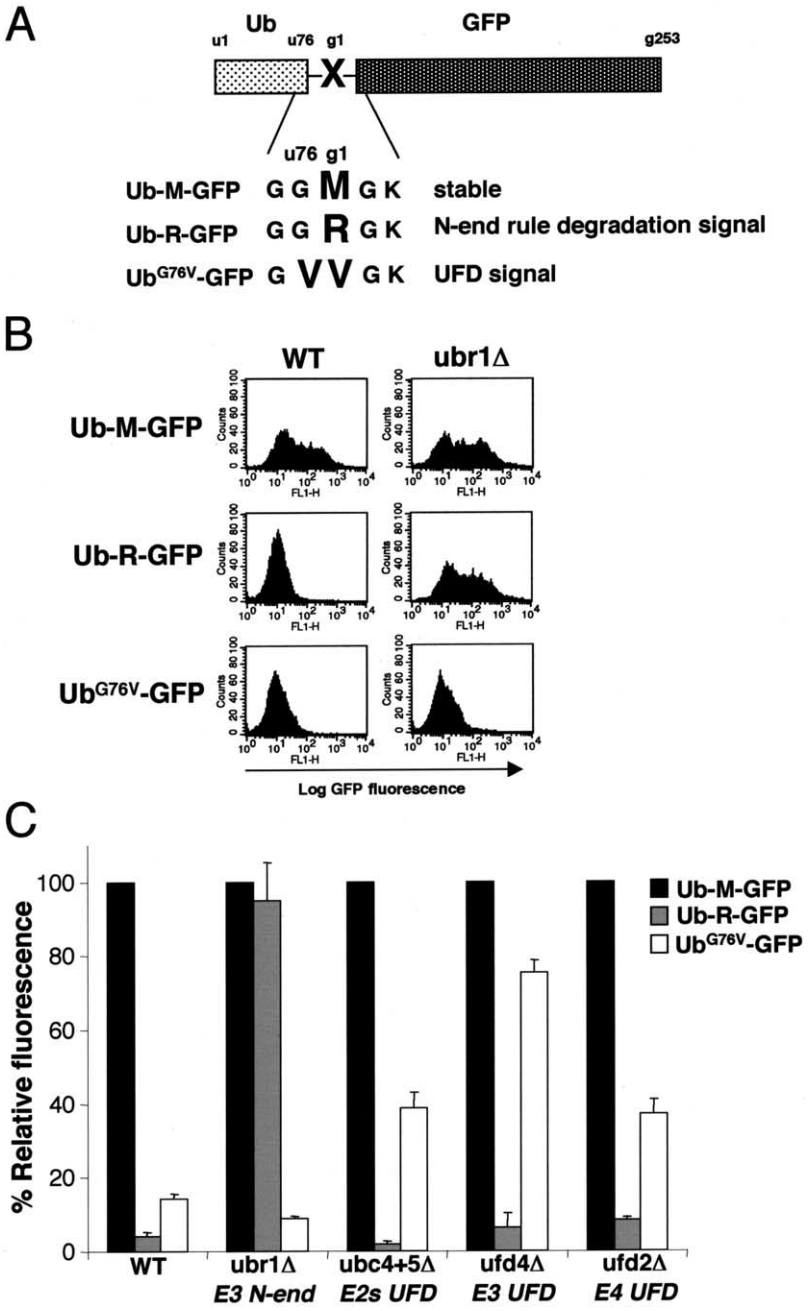

Fig. 1. Characterisation of N-end rule and UFD GFP substrates in yeast. A: Schematic drawing of the ubiquitin–GFP fusions. The linker amino acid sequences of the stable Ub-M-GFP, the N-end rule Ub-R-GFP and the UFD Ub^{G76V}-GFP constructs are depicted. B: Flow cytometric analysis of the GFP levels in wild type yeast and ubr1Δ mutant transformed with Ub-M-GFP, Ub-R-GFP and Ub^{G76V}-GFP. C: Quantification of the relative fluorescence intensities of wild type and mutant yeast strains transformed with Ub-M-GFP, Ub-R-GFP and Ub^{G76V}-GFP constructs. Mean fluorescence intensities were measured with flow cytometry and standardised with Ub-M-GFP in the corresponding strain as 100%.

in the ubr1Δ strain, resulting in fluorescent intensities comparable to those observed with the stable Ub-M-GFP while the fluorescence remained at background levels in ubr1Δ expressing Ub^{G76V}-GFP (Fig. 1B,C). Conversely, Ub^{G76V}-GFP gave increased fluorescence intensities in the ubc4/ubc5Δ, ufd4Δ and ufd2Δ mutants (Fig. 1C), although the fluorescent intensity did not reach the levels achieved with the stable Ub-M-GFP. The ubc4/ubc5Δ double mutant reached the same fluorescent intensity of the E4 ufd2Δ mutant while deletion of the E3 UFD4 had a stronger stabilising effect (Fig. 1C). As expected, the Ub-R-GFP did not show increased fluorescence in these mutants. Together, these data confirm that independent ubiquitination pathways target the Ub-R-GFP and Ub^{G76V}-GFP reporters.

3.2. *The GA repeat inhibits ubiquitin-dependent proteolysis in yeast*

In order to investigate the effect of the EBV GA repeat in yeasts, the 239-amino acid repeat of the prototype B95.8 EBV strain [34] was inserted at the C-terminus of the Ub-M-GFP and Ub-R-GFP reporters. Unexpectedly, insertion of the repeat resulted in a strong reduction in the steady-state levels of

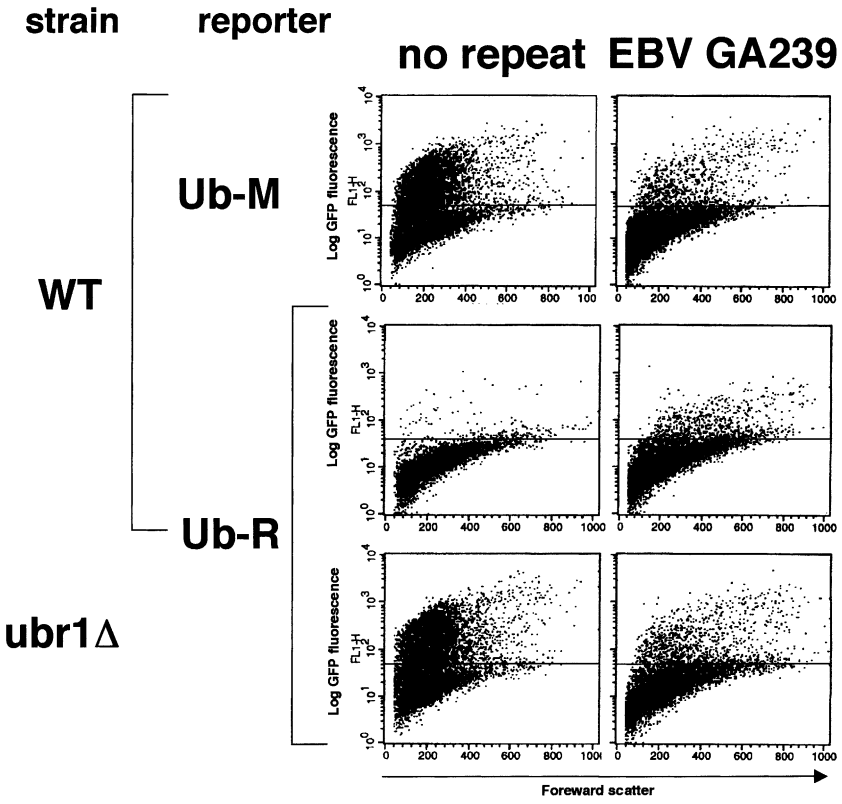

Fig. 2. Expression of EBV GA repeat fusions in yeast. Flow cytometric analyses of wild type and ubr1Δ mutant yeast expressing Ub-M-GFP and Ub-R-GFP with and without the EBV GA repeat of 239 amino acids.

both the stable Ub-M-GFP (Fig. 2, upper panel) and the unstable Ub-R-GFP reporter in the ubr1Δ mutant (Fig. 2, lower panel). A small but reproducible increase in fluorescence was observed with the Ub-R-GFP-GA compared to Ub-R-GFP in wild type yeast in line with a possible stabilising effect of the repeat (Fig. 2, middle panel).

The low fluorescence of the stable Ub-M-GFP-GA suggests that insertion of the viral sequence may directly affect the expression of the fusion protein, thereby masking the effect of the repeat. Indeed, the glycine residues in the EBV repeat are encoded by GGG or GGA codons and the alanine by the GCA codon, which have a low usage frequency (11, 21 and 11%, respectively) in highly expressed yeast genes (ftp://genome-ftp.stanford.edu/pub/codon/ysc.gene.cod). To test whether the GA codon usage may influence expression, we used a sequential cloning strategy to generate a rGA repeat with a yeast codon-optimised sequence (Fig. 3A,B). In this repeat, the glycine and alanine residues are encoded by high frequency GGT (49%) and GCT (38%) codons, respectively. The rGA contains multiple copies of the minimal repeat that was shown to be protective in mammalian cells, Gly-Gly-Ala-Gly-Ala-Gly-Ala-Gly [19], interspersed by single serine residues that were introduced for cloning purposes. The serine residues are not present in the EBV repeat but are found in the repeats of EBNA-1 homologues from primate viruses [35]. In order to validate the recombinant sequences, rGA repeats of 53, 107 and 215 amino acids were first checked for their capacity to reproduce the effect of the viral GA repeat in mammalian cells. HeLa cells were transiently transfected with Ub-M-GFP and Ub-R-GFP carrying no repeat, rGA215 or EBV GA239 and expression was monitored by Western blot (Fig. 4A) or transfected cells were treated with proteasome inhibitor and analysed by flow cytometry (Fig. 4B). In accordance with previous results [29], treatment of Ub-R-GFP-transfected cells with 10 μM Z-L_3-VS induced

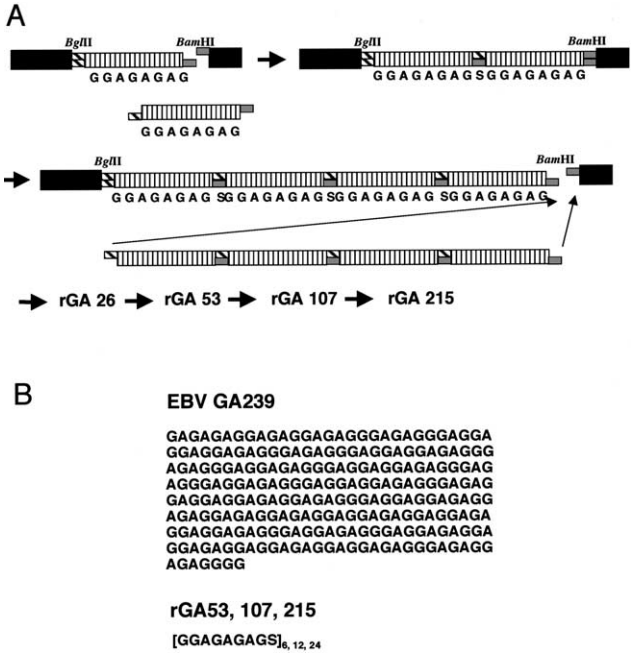

Fig. 3. Generation of yeast codon-optimised rGA repeat. A: Schematic presentation of the cloning strategy for the generation of a yeast codon-optimised rGA repeat. B: Amino acid sequences of the EBV GA239 and the rGA repeats.

a clear increase in the percentage of fluorescent cells (Fig. 4B). Insertion of either the rGA215 or the EBV GA239 resulted in increased steady-state levels of Ub-R-GFP and a high percentage of fluorescent cells with minimal further increase upon inhibition of the proteasome (Fig. 4A,B). Although complete protection was not achieved, the comparable ratio of fluorescent cells in the absence and presence of inhibitor confirms that the EBV GA239 and the rGA repeats are equally capable of blocking proteasomal degradation in mammalian cells (Fig. 4C).

In agreement with the possibility that the low expression of the fusion proteins containing the viral GA repeat was primarily due to poor synthesis, Ub-M-GFP fusions containing the yeast codon-optimised repeats rGA53, rGA107, rGA215 were clearly detected in yeast although a rGA length-dependent decrease in the expression levels of the chimeras was still observed (Fig. 5A). We then analysed the fluorescence intensities of yeast expressing Ub-R-GFP and Ub^{G76V}-GFP with and without rGA repeats. Representative fluorescence histograms of yeast cells expressing Ub-R-GFP with and without the rGA are shown in Fig. 5B and the summary of results obtained in three independent experiments performed with each construct is shown in Fig. 5C. To compensate for the length-dependent decrease in the steady-state levels, the data in Fig. 5C were normalised to the fluorescence intensity of yeast expressing Ub-M-GFP fusions containing the same re-

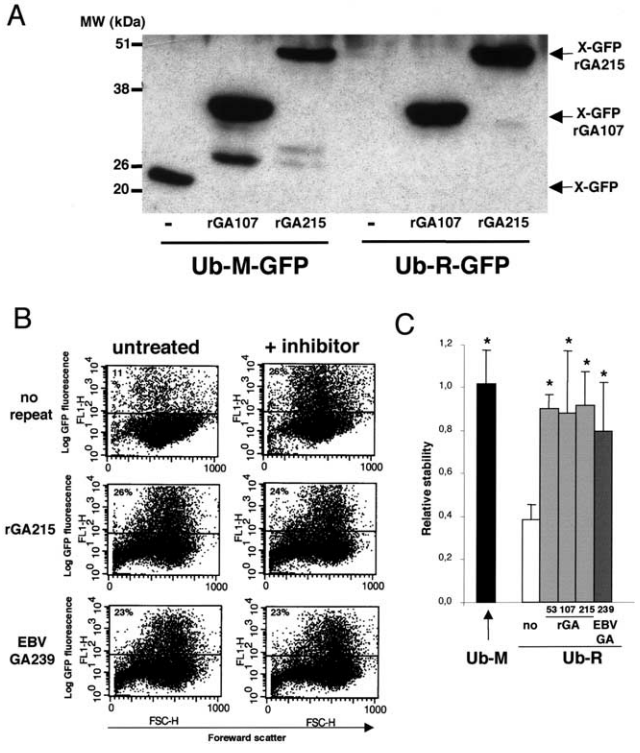

Fig. 4. The rGA repeat is functional in mammalian cells. A: HeLa cells were transiently transfected with Ub-M-GFP or Ub-R-GFP without repeat or with the rGA107 or rGA215. Lysates were analysed by Western blotting using a polyclonal GFP antibody. B: Transiently transfected HeLa cells were incubated with 10 μM of the proteasome inhibitor Z-L_3-VS or left untreated. Ten hours after administration of the inhibitor the cells were harvested and analysed by flow cytometry. The percentage of GFP-positive cells is indicated in the upper left corner. C: Quantitative analysis of flow cytometric analysis as depicted in B. Relative fluorescence is expressed as the percentage of GFP-positive cells in the absence of inhibitor divided by the percentage of GFP-positive cells in the presence of inhibitor. Values are mean and standard deviations of three independent experiments. Values significantly different from Ub-R-GFP are indicated with an asterisk (*t*-test, $P < 0.01$).

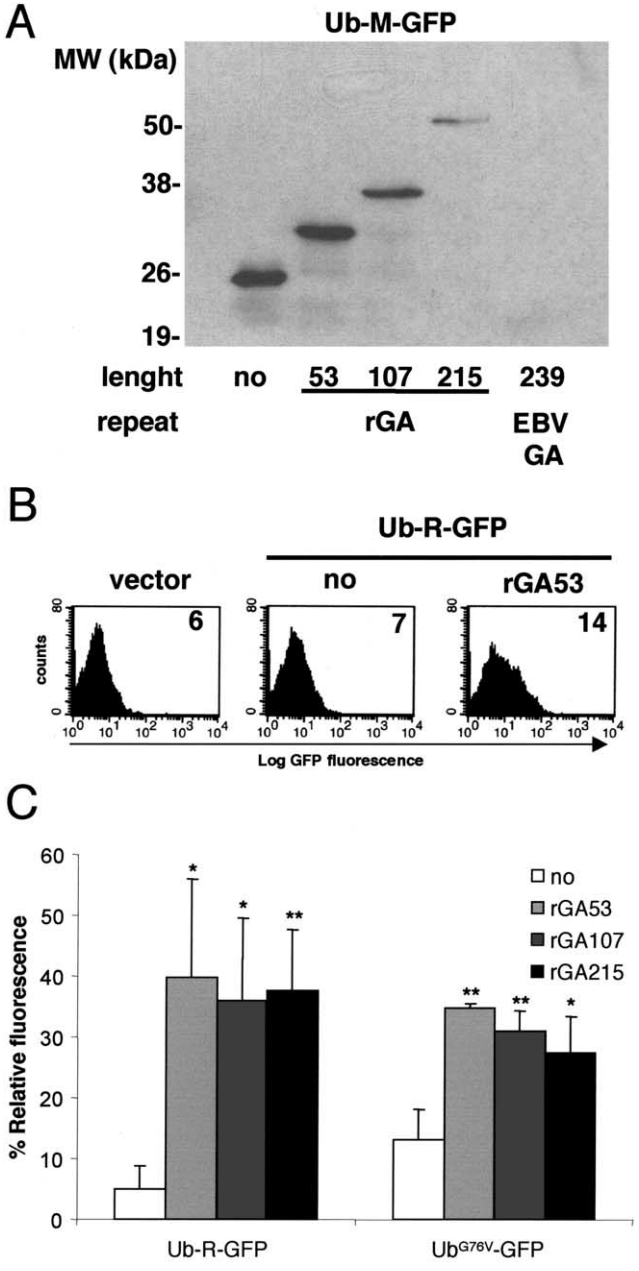

Fig. 5. Expression of rGA fusions in yeast. A: Expression of the indicated Ub-M-GFP-rGA fusions was induced in wild type yeast. Lysates were analysed by Western blot analysis with a polyclonal anti-GFP antibody. B: Representative flow cytometry analyses illustrating the stabilising effect of the rGA on Ub-R-GFP. Mean fluorescence intensities are indicated in the upper right corner of each histogram. C: Relative fluorescence intensities of yeast expressing the indicated Ub-R-GFP-rGA and Ub^{G76V}-GFP-rGA fusions. The relative fluorescence was calculated by dividing the mean fluorescence intensity of Ub-R-GFP-rGA and Ub^{G76V}-GFP-rGA fusion-expressing yeast by the mean fluorescence intensity of yeast expressing the Ub-M-GFP-rGA with the same repeat length. Values are mean and standard deviations of three independent experiments. Values significantly different from the control Ub-R-GFP and Ub^{G76V}-GFP lacking the repeats are indicated with one asterisk (*t*-test, $P < 0.05$) or two asterisks (*t*-test, $P < 0.01$).

peat. Both Ub-R-GFP-rGA and Ub^{G76V}-GFP-rGA were expressed at significantly higher levels compared to their repeatless counterparts.

In order to test if the increased steady-state levels were indeed due to inhibition of ubiquitin-dependent proteolysis, the degradation of the chimeras was monitored in pulse chase experiments. The half-life of Ub-R-GFP was approximately 5 min, while introduction of the rGA107 or rGA215 resulted in half-lives of over 20 min (Fig. 6A,B). Furthermore, insertion of the rGA53 prolonged the half-life of Ub^{G76V}-GFP from approximately 20 min to full stabilisation within the analysed time interval (Fig. 6C,D). These data show that the rGA repeat can inhibit ubiquitin-dependent proteasomal degradation of N-end rule and UFD substrates in *S. cerevisiae*.

3.3. The protective effect of the rGA does not require the polyubiquitination binding subunit Rpn10

Previous studies have demonstrated that the GA repeat does not affect the ubiquitination of proteasomal substrates [20] and ubiquitinated p53-GA chimeras maintain the capacity to interact with the polyubiquitin binding proteasome subunit S5a [18]. The vast majority of the cellular S5a is not in complex with the proteasome in yeast [36], which prompted us to test whether sequestration of the polyubiquitinated GA repeat-containing proteins could be of importance for the protective effect. Deletion of the yeast S5a homologue, Rpn10, results in stabilisation of UFD substrates while degradation of

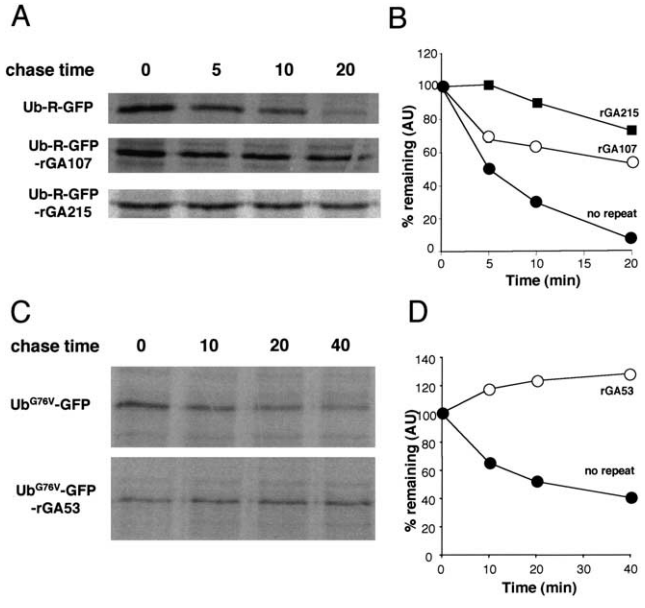

Fig. 6. The rGA repeat protects N-end rule and UFD substrates from proteasomal degradation. Expression of Ub-R-GFP and Ub^{G76V}-GFP without or with rGA repeats was induced in wild type yeast and their turnover was determined by pulse chase analysis. Yeast was labelled for 10 min with [^{35}S]Met and subsequently incubated for the indicated time points with an excess of unlabelled Met. A: Autoradiograms of pulse chase analysis of yeast expressing Ub-R-GFP (upper panel), Ub-R-GFP-rGA107 (middle panel) and Ub-R-GFP-rGA215 (lower panel). B: Densitometric quantification of the autoradiogram shown in A. C: Autoradiograms of pulse chase analysis of yeast expressing Ub^{G76V}-GFP (upper panel) and Ub^{G76V}-GFP-rGA53 (lower panel). D: Densitometric quantification of the autoradiogram shown in B. One representative experiment out of three.

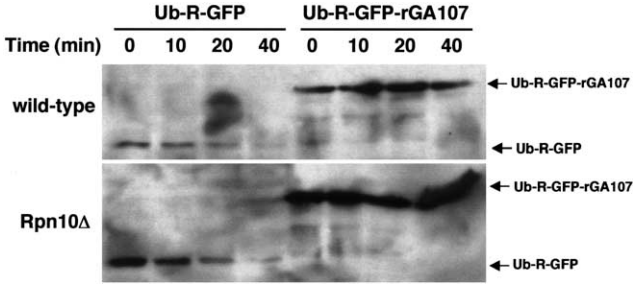

Fig. 7. Rpn10 is not required for the protective effect of the rGA repeat. Expression of Ub-R-GFP and Ub-R-GFP-rGA107 was induced in wild type yeast and the rpn10Δ strain. The GAL1 promoter was shut off by changing from galactose to glucose as a carbon source in combination with addition of cycloheximide to block translation after which the degradation of the GFP fusion was followed. Western blot analysis of Ub-R-GFP and Ub-R-GFP-rGA107 in wild type and rpn10Δ strain after promoter shut off.

N-end rule substrates is unaffected [27]. We therefore compared the degradation of Ub-R-GFP and Ub-R-GFP-rGA107 in wild type yeast and rpn10Δ strain in a promoter shut-off experiment. Ub-R-GFP was efficiently degraded in the absence of Rpn10 while the rGA-containing chimera was stable in the mutant (Fig. 7). Thus, the Rpn10 protein is not required for the protective effect of the repeat in yeast.

4. Discussion

In this study we have shown that the ubiquitin-dependent proteolysis of two GFP-based proteasome substrates in *S. cerevisiae* can be blocked by introduction of a designed rGA repeat that resembles the wild type repeat found in EBV isolates. Analysis of the degradation of UFD- and N-end rule-targeted GFP in wild type and mutant yeasts confirmed that these substrates are targeted through different ubiquitination pathways. Interestingly the Ub^{G76V}-GFP reporter was only partially stabilised in UFD deletion mutant yeasts. The UFD substrates are targeted via conjugation of separate ubiquitin chains to Lys29 and Lys48 of the N-terminal ubiquitin moiety [26]. The E2s, E3 and E4 of the pathway, UBC4/UBC5, UFD4 and E4, respectively, are differently involved in the formation of these two trees. Specifically, UBC4/UBC5 and UFD4 are important for the formation of the Lys29 tree [26], while UFD2 elongates Lys48 ubiquitin trees [2]. We have previously shown that, in contrast to previously characterised UFD substrates that require the Lys29 tree or both trees [26], Ub^{G76V}-GFP substrate can be targeted in mammalian cells independently by Lys29 or Lys48 polyubiquitin trees [37]. Hence, the partial stabilisation observed in these mutants may be due to the fact that only a single ubiquitin tree is affected in each of these mutants while the remaining tree can still trigger degradation of Ub^{G76V}-GFP.

The protective effect the rGA in yeast can be explained in two ways. The GA repeat may function as an autonomous element in a manner similar to the polyglutamine repeats found in neurodegenerative disorders, which stabilise proteins through the formation of macromolecular aggregates [38]. Alternatively, the GA repeat may target a conserved step in the proteolytic pathway that is present in both yeast and higher eukaryotes. Although we cannot formally exclude the possibility that the rGA may induce the formation of submicro-

scopic aggregates in yeasts, data from earlier studies in mammalian cells strongly support the second model. Structural analysis of an IκB-α-GA repeat chimera revealed a flexible structure for the inserted repeat [39]. Moreover, microscopic examination of cells expressing GFP-GA fusions did not reveal macromolecular aggregates of the type observed in cells expressing extended polyglutamine repeat-containing proteins [29]. In several model systems we have shown that the protective effect of the viral repeat is restricted to the harbouring protein and does not hinder proteasomal degradation of other substrates [19,20]. In contrast, polyglutamine proteins and other designed proteins with stable domains cause a general obstruction of proteasomal degradation, which is presumably caused by choking the proteasome with indigestible stable substrates [40]. The most striking evidence against the possibility that the GA repeat may act as an autonomous element comes from the in vitro degradation of model substrates where a short soluble GA repeat peptide can efficiently block ubiquitin-dependent proteolysis in a dose-dependent manner without affecting the enzymatic activity of the proteasome [23].

The demonstration that the GA repeat retains its inhibitory activity in yeast opens the possibility for a detailed molecular analysis of its mechanism of action based on the use of yeast mutants. We have earlier shown that, while the polyubiquitinated GA repeat-containing proteins fail to establish a stable interaction with the proteasome [19], they were still capable of interacting with the polyubiquitin binding subunit S5a/Rpn10 [20]. Since a large fraction of the intracellular S5a/Rpn10 is not in complex with the 19S cap of the proteasome [27,36], interaction with free S5a may preclude the recruitment of the substrate to the proteasome. Using a yeast Rpn10 deletion mutant, we have now conclusively demonstrated that interaction with this component of the ubiquitin–proteasome pathway is not required for the protective effect of the repeat.

The mammalian transcription factor NF-κB [41] and the related yeast proteins STP23 and MGA2 [42] are activated by partial proteasomal processing of their precursors. It has been postulated that the GRR domain that drives the processing of NF-κB may share some similarity with the EBV GA repeat [21]. The low complexity sequences present in NF-κB, STP23 and MGA2 may allow the formation of a flexible loop that is required for endoproteolytic processing of the substrate by the proteasome [22]. The GA motif contained within the GRR may further protect the part of the precursor that 'escapes' from degradation. In line with this scenario, the low complexity sequence resides on the product part of each of these precursor proteins. However, Sears and co-workers have reported that, while the NF-κB precursor is correctly processed in yeast, processing becomes independent of the GRR [43]. This suggests that subtle differences may exist in the mode of action between the repeats in mammalian and yeast cells.

We have previously postulated that the turnover of substrates of the ubiquitin/proteasome system may be determined by the balance between degradation signals, which target proteins for ubiquitination, and stabilisation signals that counter the degradation signals either by reversing the ubiquitination of proteins or by modifying a post-ubiquitination step [13]. The evidence present in this study is in line with the idea that such stabilisation signals are ancient regulatory elements that modulate ubiquitin/proteasome-dependent proteolysis.

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