Inhibition of ubiquitin-dependent proteolysis by a synthetic glycine–alanine repeat peptide that mimics an inhibitory viral sequence

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Abstract The glycine–alanine repeat (GAr) of the Epstein–Barr virus nuclear antigen-1 is a *cis*-acting transferable element that inhibits ubiquitin/proteasome-dependent proteolysis in vitro and in vivo. We have here examined the effect of a synthetic 20-mer GAr oligopeptide on the degradation of iodinated or biotin labeled lysozyme in a rabbit reticulocyte lysates in vitro assay. Micromolar concentrations of the GA-20 peptide inhibited the hydrolysis of lysozyme without significant effect on ubiquitination. Addition of the peptide did not inhibit the hydrolysis of fluorogenic substrate by purified proteasomes and did not affect the ubiquitination of lysozyme. An excess of the peptide failed to compete for binding of a synthetic tetra-ubiquitin complex to the S5a ubiquitin-binding subunit of the 19S regulator, confirming that the GAr does not block the access of ubiquitinated substrates to the proteasome. Our data suggest that the GAr may act by destabilizing the interaction of ubiquitinated substrates with the proteasome and promote the premature release of the substrate.

Key words: Epstein–Barr virus nuclear antigen-1; Gly–Ala repeat; Proteasome; S5a; Ubiquitination

1. Introduction

Ubiquitin/proteasome-dependent proteolysis is critically involved in the regulation of many cellular processes such as the cell cycle, differentiation, transcription, antigen presentation and the selective degradation of misfolded and damaged proteins [1]. The presentation of antigenic peptides derived from ubiquitin–proteasome-dependent degradation of viral proteins to MHC class I restricted cytotoxic T cells is a central component of antiviral responses [2]. It is therefore not surprising that viruses have developed means to block proteasomal processing in order to escape detection by the host immune system [3–9]. One interesting example of this strategy of immunoescape is the Epstein–Barr virus (EBV) nuclear antigen-1 (EBNA1), an essential viral product that is required for the maintenance and replication of EBV episomes in proliferating virus infected cells [10,11].

EBNA1 is composed of unique N- and C-terminal domains separated by a long internal repeat of glycine and alanine residues only (GAr) [12]. We have previously shown that the GAr delivers an inhibitory signal that interferes with the production of MHC class I restricted epitopes [11]. It also acts as a transferable element on the processing of a wide variety of proteasomal substrates including viral and cellular proteins [13–15]. Studies on the functional characteristics of the GAr have shown that the inhibitory effect is independent on the site of insertion into the target protein and occurs over a wide range of sizes of the repeat [13,14]. Of note, an eight amino acids long repeat was sufficient to protect IκBα from ubiquitin-dependent proteolysis [14,16]. The repeat does not act by inducing a random structural alteration of the target protein since chimeras containing repeats of various lengths remained functionally active, they formed complexes with their natural binding partners and were phosphorylated, ubiquitinated or translocated to the appropriate cellular compartments upon specific stimulation [14,17].

The mechanism by which the GAr protects different substrates from ubiquitin-dependent proteolysis is still poorly understood. The demonstration that GAr containing proteasomal substrates are efficiently ubiquitinated in vitro [13] and in vivo [14] suggests that the GAr affects a post-ubiquitination event. In line with this possibility, insertion of the repeat inhibited the interaction of ubiquitinated GAr containing IκBα chimeras with the proteasome as detected by co-immunoprecipitation assays [14]. This could not be ascribed to the induction of significant conformational changes since nuclear magnetic resonance and circular dichroism studies failed to detect changes in domain organization, folding or thermal stability compared to wild-type IκBα. [18]. Collectively, these findings suggest that the GAr may act either directly, by disturbing the interaction between the substrate and the 26S proteasome, or indirectly, by serving as a recognition domain for a molecular chaperone that sequesters the substrate away from the ubiquitin/proteasome pathway.

In the present investigation we have studied the effect of a synthetic GAr polypeptide in in vitro degradation assays. We reasoned that *trans*-inhibition of proteolysis by an excess of the peptide would exclude the involvement of chaperones that utilizes the GAr as a recognition signal for relocalization of the substrate. We report that addition of a 20-mer GAr blocks the degradation of a reference proteasomal substrate in a standard rabbit reticulocyte lysate in vitro degradation assay. The peptide did not affect the enzymatic activity of the proteasome nor the ubiquitination of the substrate and was unable to compete for binding of synthetic polyubiquitin conjugates to the specific recognition site in the S5a subunit of the 19S regulator.

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2. Materials and methods

2.1. Reagents

Hen egg white lysozyme was purchased from Sigma. 1-Biotinamido-4-[4′-(maleimidomethyl) cyclohexanecarboxamido]butane (Biotin-BMCC) was from Pierce. Ubiquitin aldehyde and tetra-ubiquitin were from Affiniti Research Products, Exeter, UK. The proteasome inhibitors carboxybenzyl-leucyl-leucyl-leucine vinyl sulfone (Z-L_3-VS) was a gift from H. Ploegh (Harvard Medical School, Boston, MA, USA); carboxybenzyl-leucyl-leucyl-leucinal (MG-132) was from Peptide Institute, Inc.; lactacystin was from Affiniti. The 20-mer GA-20 (AGAGGGAGGAGAGGGAGGAG), and the 15-mers FRNL (FRNLAYGRTCVLGKE) and LSRA (LSRAQQADAGGASGS) oligopeptides were produced by Alta Bioscience, the University of Birmingham, UK. Peptide stocks were kept at a concentration of 5 mg/ml in DMSO.

2.2. Labeling of lysozyme

Chicken egg white lysozyme was labeled using ^{125}Iodine (Amersham Pharmacia Biotech) and Chloramine T-catalyzed iodination reaction as described previously [19]. The peak radioactive fraction (500 μl, with protein concentration 0.75 mg/ml) was collected and used as substrate for in vitro degradation assays. Biotinylation was performed using the Biotin-BMCC sulfhydryl-reactive biotinylation reagent, according to the manufacturer's instructions. Briefly, 6 mg of lysozyme in a 2.5 ml volume of PBS were incubated with 0.1 ml of 8.5 mM Biotin-BMCC reagent in DMSO for 2 h at room temperature. The excess of Biotin-BMCC was removed by microconcentrator (Millipore) centrifugation. For in vitro degradation assays biotinylated lysozyme was chloraminated in ratio 1:1 with Chloramine-T (1 mg/ml in PBS) for 5 min and then equal volumes of 50 mM sodium bisulfite and 50 mM ascorbic acid were added to the reaction. The partially denaturated lysozyme was then washed with ascorbic acid using microconcentrator centrifugation. Stocks of labeled lysozyme at a concentration of 0.5 mg/ml were stored at a −70°C until use.

2.3. In vitro ubiquitination and degradation assays

Rabbit reticulocyte lysates were prepared as described previously [20] and used as a source of proteasomes and ubiquitination enzymes. The ubiquitination and degradation reactions were performed as described previously [13]. Briefly, 8 μl of crude rabbit reticulocyte lysate was added to a reaction mixture containing 40 mM Tris–HCl, pH 7.6, 5 mM Mg_2Cl, 2 mM DTT, 0.2 mg/ml ubiquitin and either iodinated or biotinylated protein substrate (2 μl) in a total volume of 20 μl. The reactions were carried out for 2 h at 37°C in the presence of ATP-regenerating system (0.5 mM ATP, 10 mM phosphocreatine, 0.2 mg/ml creatine phosphokinase). For ubiquitination assays, the reaction was performed for 20 min at 37°C in the presence of the isopeptidase inhibitor, ubiquitin aldehyde at a final concentration 20 μg/ml. The control reactions were kept on ice. The reactions were terminated by addition of SDS-sample buffer and the degradation products were fractionated by 12% SDS–PAGE. Specific bands were visualized either by Phosphorimager (Molecular Dynamics) analysis of dried gels for iodinated lysozyme or by NeutrAvidin, horseradish peroxidase conjugated reagent (Pierce) and enhanced chemiluminescence (SuperSignal West Dura extended duration substrate, Pierce) Western blots for biotinylated lysozyme. Where indicated, the proteasome inhibitors Z-L_3-VS, MG132, lactacystin or specific oligopeptides GA-20 and FRNL were added in ubiquitination and degradation assays.

2.4. Measurement of enzymatic activity of the 20S proteasome

20S proteasomes were purified in the absence of ATP as described previously [21]. Fluorogenic substrates succinyl-Leu-Leu-Val-Tyr-aminomethylcoumarin (Suc-LLVY-AMC), Boc-Leu-Arg-Arg-aminomethylcoumarin (Boc-LRR-AMC) and acetyl-Tyr-Val-Ala-Asp-aminomethylcoumarin (Ac-YVAD-AMC) were used to assay the chymotrypsin-like, trypsin-like and caspase-like activities of the proteasome, respectively, were from Sigma. Fluorogenic substrates (100 μM) were incubated for 1 h at 37°C with purified proteasomes in buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$ and 1 mM DTT in a final volume of 100 μl. Where indicated, the proteasomes were pre-incubated either with GA-20, FRNL and LSRA oligopeptides or proteasome inhibitor Z-L_3-VS for 30 min at 37°C before substrates were added. Reactions were quenched with 1 ml of 99% ethanol and the fluorescence was determined in a fluorimeter (Perkin-Elmer, Beaconsfield, UK) using an excitation of 380 nm and emission of 440 nm.

2.5. Production of a GST–S5a fusion protein and tetra-ubiquitin binding assay

The S5a, S8 and S12 genes were amplified from Marathon-Ready human leukocyte cDNA library (Clontech) using the sense primer: 5′-AAAAGATCTCC**ATG**GTGTTGGAAAGCACTA (*Bgl*II restriction site, underlined; start codon of S5a in bold) and the antisense primer: 5′-AAAGTCGAC**TCA**CTTCTTGTCTTCCTCCTT (*Sal*I restriction site, underlined; stop codon in bold) for S5a, the sense primer: 5′-TCTAAGATCTCC**ATG**GCGCTTGACGGACCA and the antisense primer: 5′-CCTTGTCGAC**TCA**CTTCCATAATTTCTTGA for S8, and the sense primer 5′-AAAGGATCCTC**ATG**CCGGAGCTGGCAGTGCAGAA (*Bam*HI restriction site, underlined) and the antisense primer 5′-AAAGTCGAC**TTA**CTTTTTCTCCTTTTTCTCCTC for S12 in PCR reactions using Taq DNA polymerase (Life Technologies). The PCR products were digested with *Bgl*II (*Bam*HI for S12) and *Sal*I and cloned in the *Bam*HI/*Sal*I sites of the pGEX-5X-1 vector (Pharmacia Biotech) for GST-fusion protein expression. The constructs were verified by DNA sequencing. The fusion proteins were expressed in *Escherichia coli* strain BL-21 and purified under non-denaturating conditions according to the manufacturer's recommendations. The protein concentration was measured by the BCA assay (Pierce) and the purity was assessed by fractionation in 10% SDS–PAGE and Coomassie blue staining. For in vitro binding assay, 4 μg of GST-fusion proteins were immobilized on 10 μl (bed volume) glutathione–Sepharose 4B beads (Pharmacia Biotech). The beads were washed two times with 1 ml ice-cold PBS buffer and then were incubated with 2 μg of tetra-ubiquitin for 2 h at 4°C with or without

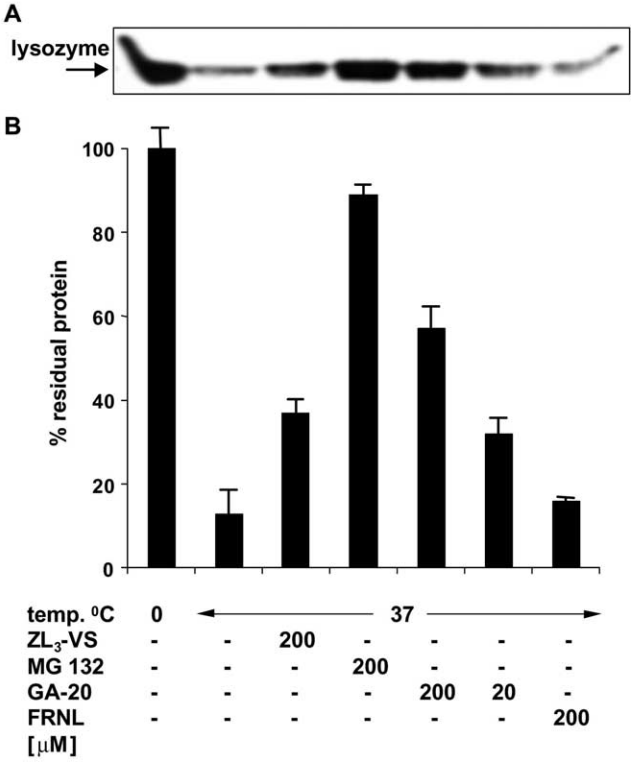

Fig. 1. A synthetic GAr peptide inhibits the degradation of iodinated lysozyme in vitro. (A) Iodinated lysozyme was mixed with crude rabbit reticulocyte lysate in the presence of ATP regenerating system and the reaction was allowed to proceed for 2 h at 37°C. The indicated amounts of Z-L_3-VS or MG-132 proteasome inhibitors, the 20 amino acids long glycine–alanine peptide (GA-20) or a 15 amino acids long peptide of random sequence (FRNL) were added to the reaction. The reaction mixture was fractionated in 12% SDS–PAGE. Control sample was kept on ice. One representative experiment out of three. (B) Densitometry analysis of the experiment presented in (A). Mean ± S.E.M. of three experiments.

addition of the indicated molar concentrations of the GA-20 or FRNL peptides. The beads were washed five times with ice-cold PBS buffer, resuspended in SDS sample buffer and fractionated by 12% SDS–PAGE. Immunoblotting was performed with an anti-ubiquitin antibody (DAKO).

3. Results

3.1. A synthetic GAr peptide blocks the ubiquitin/proteasome-dependent degradation of iodinated and biotinylated lysozyme

To investigate the mechanism by which the GAr inhibits the ubiquitin/proteasome system, a synthetic 20-mer oligopeptide containing a sequence derived from the 238 amino acid long full length GAr of the prototype B95.8 EBNA1 protein was added to in vitro degradation assays using as a substrate iodinated lysozyme. The FRNL oligopeptide of random sequence was used as a specificity control (Fig. 1A). In accordance with previous reports, iodinated lysozyme was efficiently degraded in an ATP (not shown) and temperature-dependent manner resulting in 90% decrease of the specific band detected in SDS–PAGE compared to control (Fig. 1B, lanes 1 and 2). As expected, substrate hydrolysis was efficiently blocked by addition of the proteasome inhibitors Z-L_3-VS and MG-132 (Fig. 1B, lanes 3 and 4), confirming the involvement of the proteasome. The hydrolysis of iodinated lysozyme was significantly decreased by addition of 200 or 20 μM GA-20 peptide, resulting in six- and three-fold inhibition, respectively, (Fig. 1B, lanes 5 and 6). The FRNL oligopeptide had no effect (Fig. 1B, lane 7) suggesting that the inhibitory effect is GAr peptide-specific.

In order to further analyze the mechanism of action of the GAr we developed an efficient biotinylation method for substrate labeling that provides several advantages in the in vitro assay. First, the substrate is not radioactive and the handling is therefore easier. Second, the biotin label is very stable and once the protein is labeled it may be stored for months or even years without loss of activity. Most significantly, the biotin–avidin detection method is extremely sensitive. As shown in Fig. 2A, loading of 1 ng of biotinylated lysozyme in SDS–PAGE was sufficient for a very strong signal (Fig. 2A) while up to a 10-fold higher amount of iodinated material was needed for detection (not shown). It is noteworthy that Chloramine T is used in the iodination reaction and this results in partial denaturation of the substrate. We found that this denaturation step is essential for in vitro degradation of lysozyme by the ubiquitin/proteasome system since only Chloramine T-treated biotinylated lysozyme was efficiently degraded in vitro (Fig. 2B).

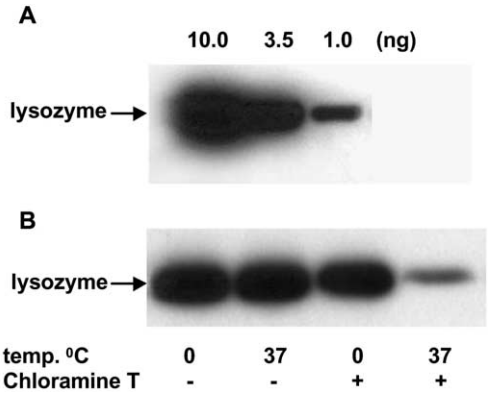

Fig. 2. Partial denaturation is required for in vitro degradation of biotinylated lysozyme. (A) The indicated amounts of biotinylated lysozyme were fractionated by 12% SDS–PAGE and the protein was blotted on nitrocellulose filter and detected by NeutrAvidin. The efficiency of detection was approximately 10-fold higher to compare to iodination. (B) Biotinylated lysozyme was exposed to crude rabbit reticulocyte lysate with or without previous treatment with Chloramine T. Only the denaturated protein was sensitive to ubiquitin–proteasome-dependent degradation.

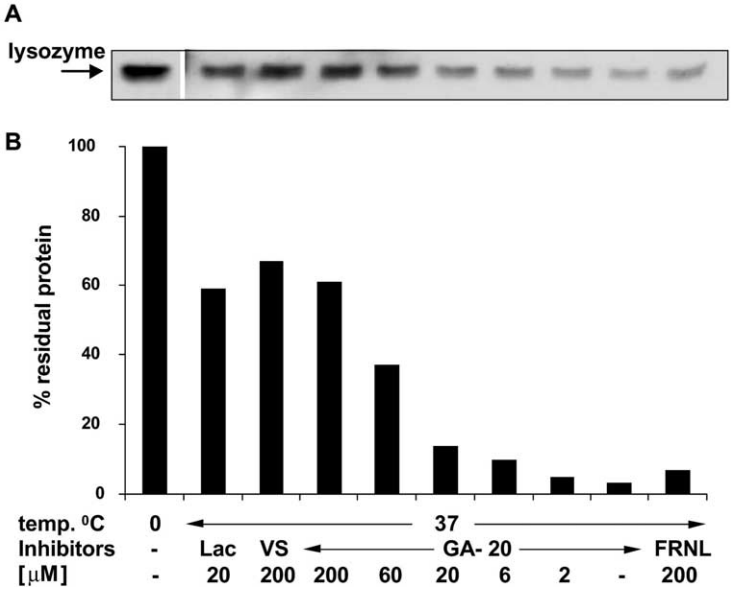

Fig. 3. The inhibition of ubiquitin–proteasome-dependent degradation by GA-20 peptide is dose dependent. (A) Biotinylated lysozyme was degraded in the presence of the indicated amounts of lactacystin or Z-L_3-VS proteasome inhibitors, the GA-20 peptide or 15 amino acid long peptide of random sequence (FRNL). The reaction mixture was fractionated in 12% SDS–PAGE. SDS–PAGE of one representative experiment out of three. (B) Densitometry analysis of the experiment presented in (A). The percent of residual protein was calculated as the ratio between the experimental sample and the control sample kept at 0°C

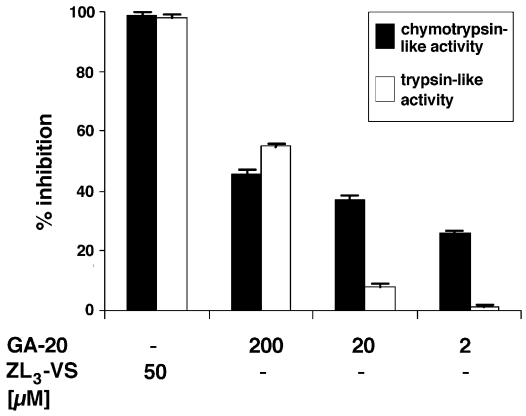

Fig. 4. The GA-20 peptide does not affect the enzymatic activity of the proteasome against fluorogenic substrates. The hydrolysis of the fluorogenic substrates Suc-LLVY-AMC (chymotrypsin-like activity) and Boc-Leu-Arg-Arg-aminomethylcoumarin (Boc-LRR-AMC) (trypsin-like activity) by semipurified 20S proteasomes was tested with or without addition of 50μM of the proteasome inhibitor Z-L_3-VS or the indicated amounts of the GA-20 peptide. The results are expressed as percent inhibition calculated as the ratio between the percent hydrolysis in the presence or absence of the indicated concentrations of the proteasome inhibitor or GA-20 peptide. The assay was calibrated in order to achieve linear dose response conditions. Mean of two experiments.

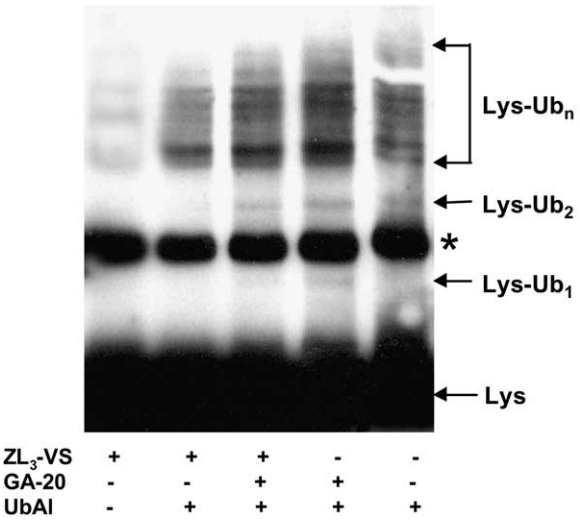

Fig. 5. The GA-20 peptide does not prevent the ubiquitination of biotinylated lysozyme. Biotinylated lysozyme was incubated in the presence of crude rabbit reticulocyte lysate, ubiquitin aldehyde (20 μg/ml), proteasome inhibitor Z-L_3-VS (200 μM) and GA-20 peptide (200 μM). The proteins were separated by 12% SDS–PAGE, transferred on to nitrocellulose membrane and detected by NeutrAvidin and enhanced chemiluminescence Western blotting. SDS–PAGE of one representative experiment out of three. The non-specific band indicated by an asterisk was detected only in this assay and is probably due to the presence of impurities in the original material that was biotinylated.

The degradation of biotinylated lysozyme was inhibited by the GA-20 peptide in a dose-dependent manner (Fig. 3A). Of note, the level of inhibition achieved in the presence of 200 μM peptide was comparable to that achieved in the presence of a similar molar concentration of Z-L_3-VS or 20 μM lactacystine (Fig. 3B).

3.2. The GAr peptide does not affect the enzymatic activity of the proteasome

To test whether the effect of the GA-20 peptide may be due to interference with the enzymatic activity of the 20S catalytical core particle, different concentrations of the peptide were included in enzymatic assays where the activity of semipurified 20S proteasomes was tested on fluorogenic substrates that monitor chymotrypsin, trypsin and caspase-like activities (not shown). While hydrolysis of the substrates was abrogated by addition of 50 μM of peptide vinyl sulfone Z-L_3-VS, the GA-20 peptide induced some inhibition only at the highest concentrations tested (Fig. 4), which is likely to reflect a non-specific interference by the high concentration of peptide. It is noteworthy that the FRNL oligopeptide could not be used as control in this assay due to the presence of both trypsin and chymotrypsin cleavage sites. (Data not shown).

3.3. The GAr peptide does not affect ubiquitination

We have previously shown that insertion of the GAr in proteasomal substrates did not affect their ubiquitination in vitro and in vivo [13,14]. To investigate whether this is true also in the presence of a great excess of the GAr we asked whether ubiquitination might be affected by the GA-20 peptide. Biotinylated lysozyme was incubated with crude rabbit reticulocyte lysate in the presence of ubiquitin aldehyde that blocks the activity of ubiquitin isopeptidases. As expected, this resulted in a significant accumulation of high molecular weight species corresponding to ubiquitinated lysozyme (Fig. 5). No difference was observed when the GA-20 peptide was added to the reaction, thus confirming that the GAr does not interfere with the ubiquitination machinery.

3.4. The GAr peptide does not affect the recognition of polyubiquitin conjugates by the S5a subunit

In the final set of experiments we asked whether the GA-20 peptide could act by blocking the binding of ubiquitinated proteins to the 19S regulatory particle of the proteasome. This is a complex issue since several subunits of the 19S are

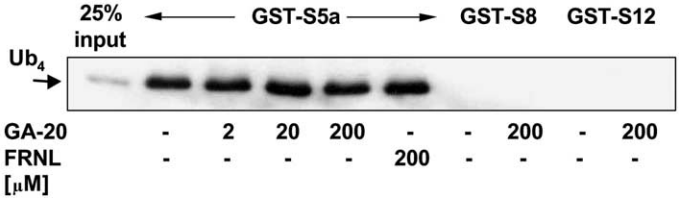

Fig. 6. The GA-20 peptide does not affect the binding of tetra-ubiquitin to S5a proteasome subunit in vitro. GST–S5a, GST–S8 and GST–S12 fusion proteins were immobilized on glutathione–Sepharose beads and then incubated with tetra-ubiquitin. Where indicated, increasing concentrations of the GA-20 peptide or the control FRNL peptide were added to the binding reaction. After extensive washing the retained proteins were fractionated by 10% SDS–PAGE and immunoblotted with anti-Ub antibody.

likely to interact with polyubiquitin, with various substrates or with substrate-bound chaperones. The best characterized interaction is that of the S5a subunit with a specific domain in polyubiquitin conjugates that contain at least four ubiquitin moieties [22–24]. In order to investigate whether the GA-20 peptide could interfere with this binding, a GST–S5a fusion protein was immobilized on glutathione–Sepharose beads and then exposed to a synthetic tetra-ubiquitin multimer that mimics the ubiquitin ladder of polyubiquitinated proteins. GST fusions of two additional components of the 19S cap, S8 and S12, that, on the basis of available literature, are not expected to bind to polyubiquitin, were included as specificity control. Tetra-ubiquitin bound very efficiently to S5a, as expected, while there was no detectable binding to S8 or S12 (Fig. 6). The strong interaction of polyubiquitin with S5a was not affected by addition of increasing concentration of the GA-20 peptide or the control FRNL peptide. Thus, the GAr does not compete for interaction of ubiquitinated proteins with the polyubiquitin acceptor site of the proteasome.

4. Discussion

The Gly–Ala repeat of EBNA1 is the only known protein domain that acts as a modular inhibitor of the ubiquitin–proteasome system and prevents proteolysis of a broad variety of proteasomal substrates, independently on their proteasome targeting signal or type of ubiquitin ligase involved in ubiquitination. Although studies in model systems have revealed some of the chemical and structural properties that are critical for the inhibitory activity of the repeat, the mechanism of action remains still elusive. One puzzling feature emerging from studies where GAr containing proteins were over-expressed in living cells is that the repeat affected exclusively the degradation of the protein where it was inserted [11]. The failure to inhibit proteolysis in trans led to the hypothesis that the repeat may alter the structure of the substrate and/or serve as a recognition signal for chaperones that sequester the substrate away from the proteasome. Later studies have revealed that the activity of the proteasome is highly redundant in cells, probably in response to the need to clear huge amounts of substrates during stress responses [15]. Thus, even when highly overexpressed, the GAr-containing proteins may not reach the concentration required for trans inhibition of the ubiquitin/proteasome system in vivo. In this report, we have reinvestigated this question by testing the capacity of a synthetic GAr peptide to interfere with the degradation of a model substrate in vitro.

We have now shown that the GAr peptide can inhibit the degradation of a model substrate in vitro. In accordance with previous observations [11,13–15], the GAr did not affect ubiquitination of the substrate and did not alter the enzymatic activity of purified proteasomes. These findings have at least two important implications. First, the finding that the GAr can, under appropriate conditions, inhibit proteolysis in trans provides conclusive evidence against its capacity to target chaperones that sequester the substrate away from the proteasome. If this were the case, addition of a competitor peptide would not have effected the processing of a substrate that lacks the GAr domain. Second, our failure to detect any significant effect on ubiquitination, strongly support the conclusion that the GAr directly affects the interaction of ubiquitinated substrates with the proteasome.

Very little is known about the events that lead to recognition of ubiquitinated substrates by the proteasome, their unfolding and tethering into the proteolytic cavity. It seems likely that several components of the 19S regulator will contribute in what is probably a tightly regulated and perfectly timed sequence of events. Conceivably, the recognition of the ubiquitinated substrate is the first, rate-limiting step. This is partly achieved through recognition of conformational domains in the polyubiquitin tree by the S5a subunit of the 19S cap [24]. Interestingly, the ubiquitin-interacting domain of S5a is a hydrophobic region that bears some similarity to the GAr [23,25]. We have shown that the GA-20 peptide does not interfere with the binding of a synthetic tetra-ubiquitin conjugate to a GST–S5a fusion in vitro. This implies that the GAr does not prevent the interaction of the ubiquitinated substrate with proteasome through competition for binding to the ubiquitin specific subunit. However, the demonstration that deletion of the S5a subunit is not lethal in yeast [26] suggests that other components of the 19S regulatory particle must be involved in substrate recognition. This additional interaction may be required to stabilize the initial binding and retain the substrate until subsequent irreversible modifications will take place. Conceivably, weakening of this complementary anchor might result in premature release of the substrate that, through the action of cytosolic isopeptidase and chaperones, will be returned to the pool of cellular proteins.

This attractive scenario may be difficult to prove experimentally for several reasons. First, the existence of a putative complementary anchor involving direct interaction between the proteasome and the substrate is difficult to reconcile with the huge number of broadly different substrates that are degraded by the proteasome. Although the existence of proteasome subunits capable of recognizing subsets of similar substrates cannot be excluded, it is possible that interaction may be achieved through specific chaperones that escort the ubiquitinated substrate to the proteasome. The recent demonstration that some ubiquitin ligases bind, directly or indirectly, to the proteasome [27–30] suggests that the E3 themselves may mediate this complementary anchor. If so, the GAr may act either by preventing binding of the chaperone to the substrate or by interfering with the interaction between the proteasome and the E3. Second, the strict temporal sequence of the events that led to proteolysis, including recognition, deubiquitination and unfolding, and the involvement of different partners at each step, suggest that all these interactions must be short lived and of low affinity. If so, even a weak interference of the GAr with any of the partners involved could have major effects on the entire process.

In conclusion, the findings reported in this paper strongly support a model for the inhibitory activity of the GAr that involves interference with the interaction of the ubiquitinated substrate with the proteasome. The nature of this interaction and the involved partners remain an important focus for future studies.

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