VWA domain of S5a restricts the ability to bind ubiquitin and Ubl to the 26S proteasome

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ABSTRACT **The 26S proteasome recognizes a vast number of ubiquitin-dependent degradation signals linked to various substrates. This recognition is mediated mainly by the stoichiometric proteasomal resident ubiquitin receptors S5a and Rpn13, which harbor ubiquitin-binding domains. Regulatory steps in substrate binding, processing, and subsequent downstream proteolytic events by these receptors are poorly understood. Here we demonstrate that mammalian S5a is present in proteasome-bound and free states. S5a is required for efficient proteasomal degradation of polyubiquitinated substrates and the recruitment of ubiquitin-like (Ubl) harboring proteins; however, S5a-mediated ubiquitin and Ubl binding occurs only on the proteasome itself. We identify the VWA domain of S5a as a domain that limits ubiquitin and Ubl binding to occur only upon proteasomal association. Multiubiquitination events within the VWA domain can further regulate S5a association. Our results provide a molecular explanation to how ubiquitin and Ubl binding to S5a is restricted to the 26S proteasome.**

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INTRODUCTION

Delivery to the proteasome of designated proteins marked for degradation by a polyubiquitin modification is a fundamental process in maintaining homeostasis in the eukaryotic cell. Failure to properly deliver such substrates gives rise to various pathologies (Ciechanover and Brundin, 2003). The 26S proteasome is composed of a 20S catalytic particle (CP) and a 19S regulatory particle (RP) that regulates 20S opening, substrate deubiquitination, and recognition. Ubiquitin recognition by the proteasome is mediated by several stoichiometric proteasomal subunits—Rpt5 (Lam *et al.*, 2002), Rpn10/S5a (Deveraux *et al.*, 1994), and Rpn13 (Husnjak *et al.*, 2008). However, most of the ubiquitin-binding ability of the proteasome is eliminated upon perturbation of Rpn10 and Rpn13 ubiquitin-binding capabilities (Husnjak *et al.*, 2008). Of the proteasomal ubiquitin receptors, Rpn10/S5a is unique with respect to its cellular distribution, as it is the only stoichiometric proteasomal subunit found outside the proteasome (van Nocker *et al.*, 1996; Matiuhin *et al.*, 2008). Rpn10/S5a binds the 26S proteasome via its N-terminal von Willebrand factor type A (VWA) domain and has a flexible linker and two ubiquitin-interacting motifs (UIMs) at the C-terminus (Riedinger *et al.*, 2010). In this respect, Rpn10/S5a is similar to several substoichiometric proteasomal ubiquitin shuttle factors (such as Rad23 and Dsk2) that contain both proteasome- and ubiquitin-binding capacities and are found both inside and outside the proteasome (Saeki *et al.*, 2002; Hartmann-Petersen *et al.*, 2003). Early observations indicated that imbalanced ratios between free and proteasomal bound Rpn10 can perturb proper substrate processing by the proteasome (Verma *et al.*, 2004). However, the question of substrate recruitment to the proteasome and its regulation with respect to the nonbound Rpn10/S5a isoform has not been addressed.

The role of the ubiquitin receptor S5a/Rpn10 in substrate degradation by the 26S proteasome has been mainly examined by work in yeast. However, the yeast and mammalian homologues carry distinct features and null phenotypes. Whereas *rpn10*Δ yeast strains have mild phenotypes (van Nocker *et al.*, 1996), knockout of the S5a

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Abbreviations used: CP, catalytic particle; HMW, high molecular weight; IP, immunoprecipitation; ITC, isothermal titration calorimetry; kd, knockdown; LMW, low molecular weight; RP, regulatory particle; ubl, ubiquitin-like; UIM, ubiquitin-interacting motif; UPS, ubiquitin proteasome system; VWA, von Willebrand factor type A.

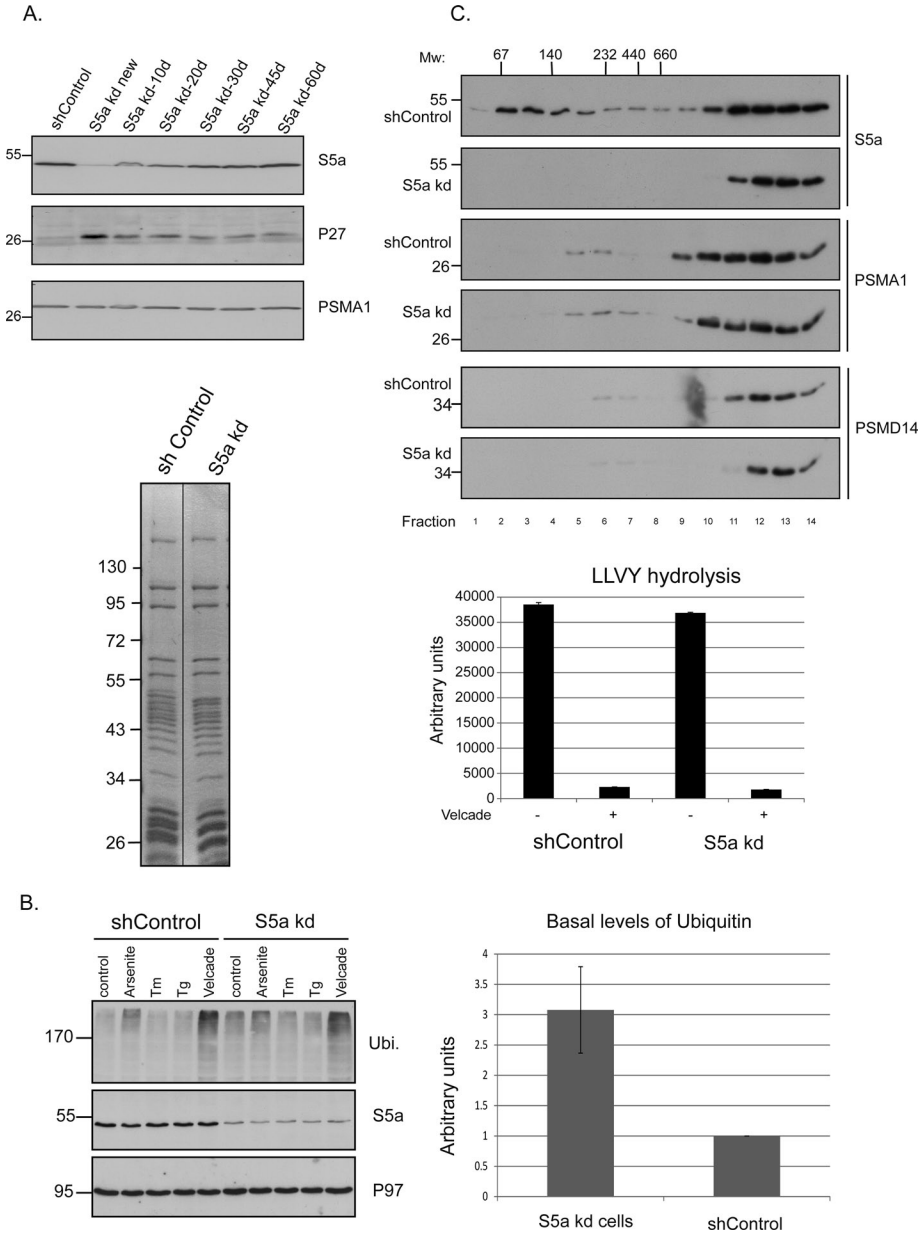

FIGURE 1: S5a knockdown cells. (A) Total cell lysates from control and S5a kd cells (continuously cultured as indicated in the text) were analyzed by immunoblotting against S5a, p27Kip1, and PSMA1. In addition, proteasomes were affinity purified from both control and S5a kd cells, and protein content was evaluated by Coomassie staining or evaluated by LLVY-AMC hydrolysis in the presence or absence of Velcade (10 μM). (B) S5a kd and control cells were treated with stress agents and subjected to ubiquitin, S5a, and P97 (serving as a loading control) immunoblots. The stress agents indicated are arsenite (0.5 mM, 1 h), tunicamycin (Tm; 20 μg/ml, 1 h), thapsigargin (Tg; 2 μM, 1 h), and the proteasome inhibitor Velcade (10 μM, 1 h). Quantification from three independent experiments indicates threefold increase of basal polyubiquitin levels in the S5a kd cells. (C) Endogenous 19S (PSMD14 or S5a) and 20S (PSMA1) distributions on glycerol velocity gradient show the HMW distribution of S5a upon S5a knockdown. A 5× quantity from the S5a kd cells was loaded on the S5a immunoblot in order to reveal any S5a reactivity in the LMW fractions.

mouse gene *(PSMD4)* is embryonic lethal at early stages of development (Hamazaki *et al.*, 2007), making it difficult to create cellular models for studying mammalian S5a. Furthermore, rescue experiments in yeast indicated the ability of Rpn10 derivatives that are unable to bind ubiquitin to rescue the *rpn10*-null phenotypes (Fu *et al.*, 1998). However, similar mutants were not sufficient to rescue mice null phenotypes (Hamazaki *et al.*, 2007), further raising the differences between yeast and mammal. Multiubiquitination on Rpn10 has been reported in yeast to regulate Rpn10 ability to recruit substrates to the 26S proteasome (Crosas *et al.*, 2006; Isasa *et al.*, 2010), in *Drosophila* to regulate ubiquitin receptors (Lipinszki *et al.*, 2012), and in humans to regulate proteasome activity (Jacobson *et al.*, 2014).

Here we show that mammalian S5a is required for proper proteasomal recruitment of polyubiquitinated substrates and ubiquitin-like (Ubl) harboring proteins and demonstrate that this interaction is favored by the proteasome bound S5a. We show that the VWA domain of S5a inhibits the ability of S5a to bind ubiquitin and Ubl when not bound to the proteasome, thus providing a mechanistic explanation that ensures proper delivery of proteasomal substrates only to the proteasomal bound S5a. We further demonstrate that multiubiquitination events within the S5a VWA domain may further regulate the ability of S5a to bind Ubl domains.

RESULTS

S5a-knockdown cells

To evaluate the cellular role of S5a and to address the cellular consequences of S5a impairments, we established a cell line in which we used RNA interference (RNAi) to obtain >90% reduction in S5a expression (Figure 1A). On continuous culturing of the established cell line (S5a kd), we noted that the cells adapted to the knockdown by overexpressing S5a, thus compensating for the reduced levels of S5a (Figure 1A). This finding reflects the fact that no compensation for the reduction of S5a was established in the S5a kd cells at early time points; thus changes observed in these cells are a direct consequence of S5a reduction and not part of a compensatory response to the reduction. This can also be observed by the elevated basal levels of p27Kip1 in nonsynchronized S5a kd cells, an elevation that is reduced upon S5a reappearance (Figure 1A). The high basal levels of p27Kip1 in the S5a kd cells is part of a cell cycle impairment observed in these cells (an attenuated transition into the G1 phase during normal cell cycle conditions and an increased sensitivity of these cells toward a sublethal dosage of the protein misfolding agent arsenite; Supplemental Figure S1). Subsequently all experiments in the S5a kd cells were performed on freshly thawed cells after S5a expression evaluation. Furthermore, proteasome complexes purified from control and S5a kd cells indicated complex integrity (Coomassie staining and complex sedimentation) and functionality (LLVY peptide hydrolysis); therefore S5a kd

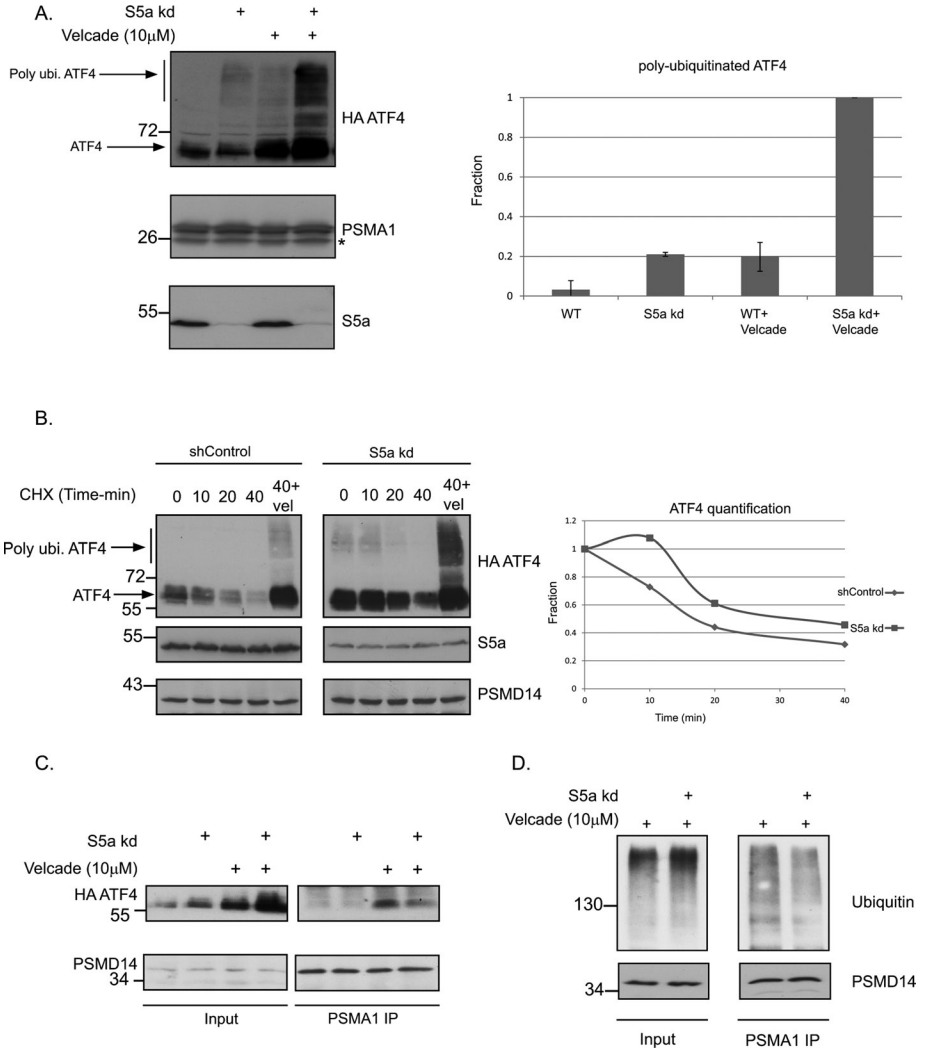

FIGURE 2: ATF4 is an S5a-dependent UPS substrate. (A) S5a kd and control cells were treated with Velcade where indicated (10 μM, 1 h) and subjected to HA, S5a, and PSMA1 (loading control) immunoblots. Quantification of the polyubiquitinated ATF4 proteasomal substrate is indicated to the right. (B) Control and S5a kd cells were evaluated toward ATF4 degradation rates by cyclohexamide treatment, and time course ATF4 content evaluation was performed by immunoblots to HA, S5a, and PSMD14. Quantification of the ATF4 content is indicated to the right, showing the delayed degradation of ATF4 in the S5a kd cells. (C) S5a kd and control cells were treated as in A and subjected to a PSMA1 IP. Proteasomal and ATF4 content were revealed by HA and PSMD14 immunoblot. (D) S5a kd and control cells were treated with Velcade and subjected to a PSMA1 IP. Proteasomal and ubiquitin content was revealed using the ubiquitin and PSMD14 immunoblots.

cells maintain an intact and functional proteasome. When challenging S5a kd cells with various protein misfolding agents (arsenite, tunicamycin, thapsigargin, or the proteasome inhibitor Velcade) we did not observe changes in polyubiquitin accumulation between S5a kd and control cells. Instead, the main observed difference was at the basal level of polyubiquitin in the nonstressed cells. Figure 1B indicates a threefold increase in the polyubiquitin signal in S5a kd cells under conditions without stress, consistent with a recent report showing higher basal levels of polyubiquitin upon S5a reduction (Sparks *et al.*, 2014). Thus S5a seems to play an important role in maintaining efficient processing of polyubiquitinated substrates by the proteasome. In yeast the Rpn10 proteasomal subunit was found in free and proteasome-bound fractions (van Nocker *et al.*, 1996; Matiuhin *et al.*, 2008). The S5a kd cells were further characterized with respect to the distribution of S5a in glycerol velocity gradients. On evaluating S5a distribution in the S5a kd cells, we did not observe S5a presence in proteasome-free fractions in spite of the excess material loaded on the S5a kd cell lysate gradient (Figure 1C). These results imply that proteasome-bound S5a is a stable component of the proteasome complex that does not exchange with the proteasome-"free" isoform, consistent with its stoichiometric presence in the proteasome (Berko *et al.*, 2014).

S5a-dependent ATF4 and polyubiquitinated substrate degradation

To address the role of S5a in polyubiquitinated substrate degradation, we took advantage of a previous report in yeast showing impaired degradation of Gcn4 in *rpn10*Δ strains (Mayor *et al.*, 2005). ATF4 is the functional mammalian homologue of Gcn4 (Harding *et al.*, 2000) and is also subject to ubiquitin-proteasome system (UPS)–dependent degradation (Lassot *et al.*, 2001). We therefore tested whether ATF4 degradation is S5a regulated. To this end, we evaluated control and S5a kd cells expressing ATF4 for their ATF4 content under normal and proteasomal inhibitory conditions. As seen in Figure 2A, Velcade treatment enabled detection of the otherwise undetectable polyubiquitinated ATF4. In contrast, polyubiquitinated ATF4 could be detected in the S5a kd cells even without Velcade treatment, implying a functional role of S5a in efficient ATF4 degradation (Figure 2A, right, quantifications). The extended half-life of ATF4 in the S5a kd cells is revealed in cycloheximide experiments, in which the consequences of the slower rate of decay are seen even at the initiating time point (Figure 2B). To evaluate whether the impaired ATF4 degradation observed in the S5a kd cells is due to impaired proteasomal recruitment or inefficient proteasomal processing of the polyubiquitinated ATF4, we immunopurified proteasomes from both control and S5a kd cells and evaluated their ATF4 content. As observed in Figure 2C, in spite of higher ATF4 levels in the S5a kd cells (input), upon proteasome purification, reduced ATF4 levels are obtained in S5a kd cells, implying a recruitment role for S5a toward ATF4. The recruitment impairment was also observed when we monitored general proteasomal polyubiquitin content. We detected lower polyubiquitin levels upon proteasomal purifications in S5a kd cells in spite of their higher levels in the cell (Figure 2D, input vs. PSMA1 immunoprecipitation [IP]). This result implies a recruitment role for S5a toward polyubiquitinated substrates, as expected from a ubiquitin-binding proteasomal component.

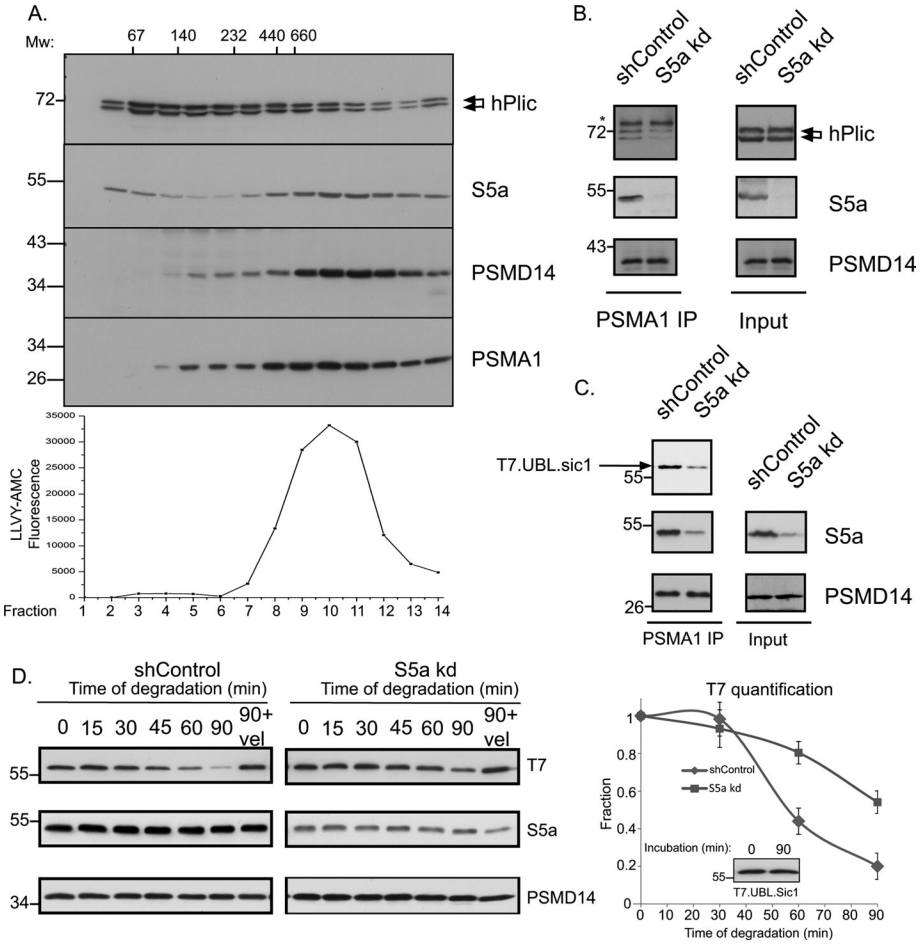

FIGURE 3: S5a–UBL interaction. (A) Immunoblot of hPlic and the proteasomal subunits PSMA1, S5a, and PSMD14 in fractions of glycerol gradients prepared from WT cells. Proteasomal chymotrypsin activity along the gradient is indicated below. (B) Lysates from control and S5a kd cells were subjected to PSMA1 (26S) IP, followed by PSMD14, S5a, and hPlic immunoblots. Asterisk indicates a nonspecific band. (C) Lysates from control and S5a kd cells treated with Velcade were subjected to PSMA1 (26S) IP, followed by incubation with the T7.UBL.Sic1 substrate. Unbound material was extensively washed, and matrix content was found with regard to Sic1 (T7), S5a, and proteasomal content (PSMD14). (D) The degradation of T7.UBL.Sic1 was performed by using 26S proteasomes acquired from control and S5a kd cells. At the indicated time points, samples were extracted and content analyzed by the various immunoblots. Note the disappearance of T7.UBL.Sic1 upon proteasomal incubation and the stability upon Velcade treatment. Quantification of T7 signal is indicated (right), and UBL.Sic1 stability in the absence of proteasomes is shown.

S5a-dependent Ubl recruitment

To further evaluate the role of S5a as a proteasomal Ubl recruitment factor, we affinity purified 26S proteasomes from control and S5a kd cells and evaluated them for the Ubl-dependent shuttling factor hPlic-2 (UBQLN2; the mammalian homologue of yeast Dsk2; Kleijnen *et al.*, 2000). As noted in Figure 3A, most hPlic is not associated with higher–molecular weight (HMW) proteasomal fractions, consistent with its role as a proteasome shuttle factor (Kleijnen *et al.*, 2003). To evaluate the dependence of hPlic on S5a proteasomal recruitment, proteasomes purified from control and S5a kd cells were evaluated for their hPlic content. A significant reduction in hPlic content was observed in the S5a kd cells (Figure 3B), implying a central role for S5a in Ubl proteasomal recruitment. To extend this evaluation to additional Ubl's, we used the ability of fusion proteins with a Ubl domain to enable proteasomal degradation (Kraut and Matouschek, 2011). We fused a Ubl domain from RAD23 to Sic1 and monitored the ability of the Ubl domain to enable proteasomal binding and degradation. On incubation of the Ubl-Sic1 substrate with proteasomes purified from control and S5a kd cells, we noted reduced binding of the substrate to the S5a kd proteasomes (Figure 3C). The outcome of the reduced proteasomal binding of Ubl in the S5a kd cells is reflected in an in vitro degradation assay in which the attenuated degradation rates of the Ubl substrate are observed in the S5a kd cells (Figure 3D).

Our data thus far indicate that Ubl proteasomal recruitment is mediated by S5a (Figure 3). To evaluate whether preferential binding of hPlic to proteasomal S5a exists, we examined whether the S5a-hPlic interaction that is observed on proteasomes (Figure 3B) can also occur in the absence of proteasomes. In spite of the low levels of hPlic in the proteasomal fractions (Figures 3A and 4A, long hPlic exposure), we were able to affinity purify hPlic from soluble proteasomes and detected S5a presence in this copurification (Figure 4A). However, no interaction between hPlic and S5a was detected in the absence of proteasomes, in spite of the high abundance of hPlic in these fractions (Figure 4A). To confirm that the lack of S5a binding to hPlic in the absence of proteasomes is not a due to the low quantities of S5a in these fractions (Figures 1C and 4B), we overexpressed S5a to increase its abundance in the lower–molecular weight (LMW) fractions. In spite of the observed increase (Figure 4B), we were unable to detect S5a presence upon hPlic immunoprecipitation (Figure 4B). We therefore conclude that S5a interaction with hPlic occurs only on the proteasome.

S5a ubiquitin binding is inhibited by the VWA domain

How might S5a prefer binding to Ubl and ubiquitin only in its proteasome-bound state? Several catalytic activities of the proteasome are restricted to occur only in the intact assembled proteasome (Yao and Cohen, 2002; Yao *et al.*, 2006; Verma *et al.*, 2004; Murata *et al.*, 2009; Lee *et al.*, 2010). Several of these restriction mechanisms include an intramolecular fold that upon proteasomal binding enables catalytic activity (Hamazaki *et al.*, 2006; Yao *et al.*, 2006). To evaluate whether S5a binding to ubiquitin is restricted to the proteasome, we deleted the N-terminal VWA domain from S5a, thus excluding the protein's distribution from the proteasome (Fu *et al.*, 1998). As shown in Figure 5A, expression of wild-type (WT) but not a UIM mutant S5a (S5a A219/290Q) in S5a kd cells reduced polyubiquitin levels. In contrast, S5aΔVWA expression induced polyubiquitin levels. On proteasomal purification (PSMA1 IP), we observe elevated levels of S5a in the proteasome (WT and UIM mutant) but not S5aΔVWA, as expected. Furthermore, the expression of S5aΔVWA seemed to reduce the amount of polyubiquitin bound to proteasomes in spite of their higher level upon S5aΔVWA

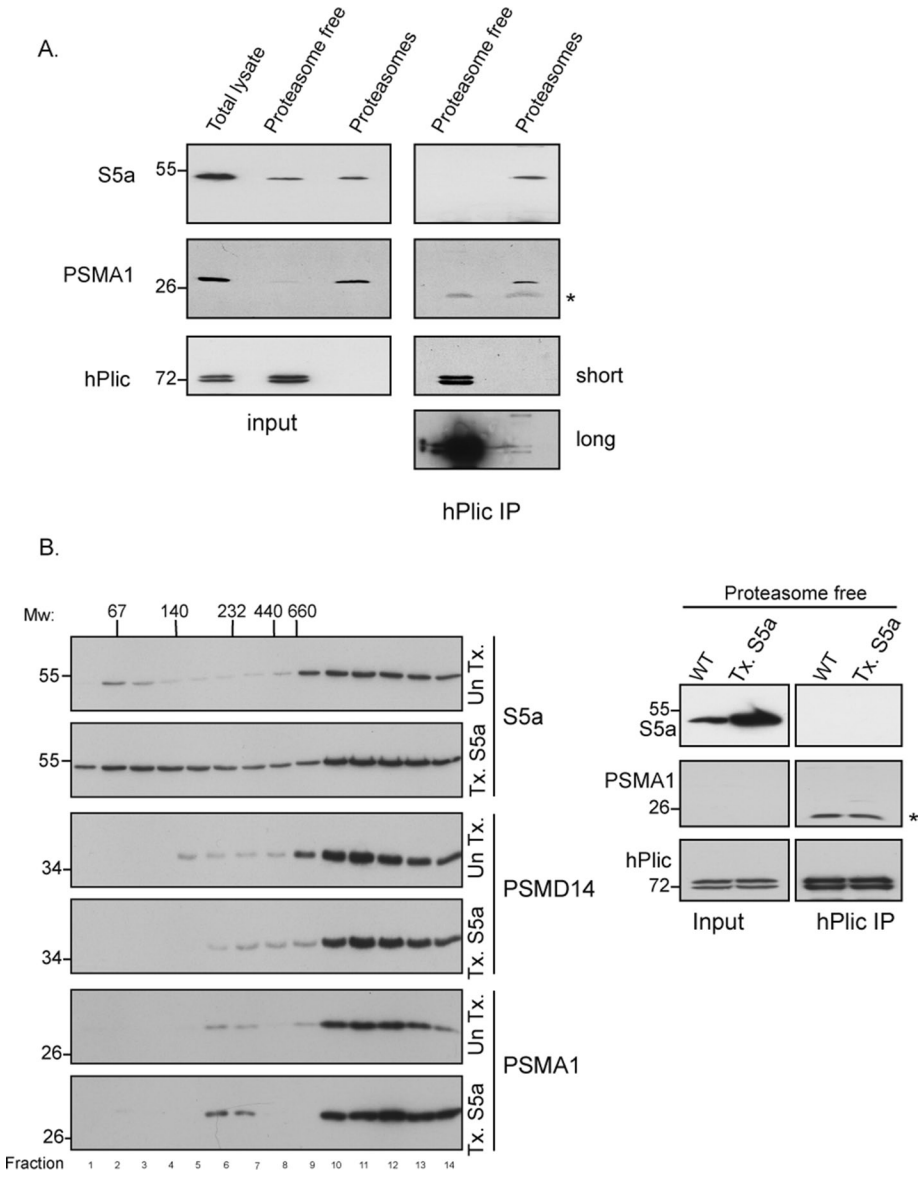

FIGURE 4: S5a–hPlic interaction in the presence of proteasomes. (A) Soluble proteasomes were acquired from WT cells by performing PSMA1 IP, followed by a peptide elution. Cell lysates depleted from proteasomes were acquired by ultracentrifugation, precipitating the HMW 26S particle. Both sources of S5a (proteasomal and free) were directly evaluated with regard to their S5a, PSMA1, and hPlic content (input) or subjected to hPlic IP. Although proteasome fractions had only small quantities of hPlic (hPlic IP, long exposure), they did reveal the presence of S5a in the hPlic IP. Asterisk, light chain, immunoglobulin G (IgG). (B) WT or S5a-overexpressing cells were evaluated for their S5a distribution. Note the high S5a content in the LMW fractions upon S5a overexpression. Proteasome free lysates obtained from untransfected (un-Tx.) and transfected S5a cells (Tx. S5a) were subjected to hPLic IP, and in spite of the abundant S5a content in the proteasome-free fraction, S5a content was not revealed in the hPlic IP. Asterisk, light chain, IgG.

expression (Figure 5A; compare input to PSMA1 IP). In contrast, upon S5a purification, we noted an increase in polyubiquitin association in WT but not the UIM S5a mutant. S5aΔVWA expression increased polyubiquitin binding even further and did not show any proteasomal association (Figure 5A; S5a IP). These results indicate that S5aΔVWA expression competes with the proteasome for binding to polyubiquitinated proteins. Therefore, in addition to its role as a proteasome-binding domain, the VWA domain has an inhibitory role toward S5a binding to ubiquitin.

To evaluate the effect of S5aΔVWA on a proteasomal process in a physiological context, we electroporated mouse muscles (tibialis anterior) with S5a WT– or S5aΔVWA-encoding plasmids and induced proteolysis (i.e., atrophy) by food deprivation. Food deprivation reduces anabolic signaling pathways in skeletal muscle and accelerates protein degradation primarily via the proteasome (Cohen *et al.*, 2012). In mouse muscles expressing S5aΔVWA, polyubiquitin conjugates accumulated upon fasting as compared with mock-transfected muscles, as shown by ubiquitin immunoblot (Figure 5B). This increase in ubiquitin conjugates may be attributable to impaired degradation via the proteasome. However, no change in the levels of ubiquitin conjugates was observed in muscles transfected with S5a WT from fasting mice as compared with control muscles (mock expressing). Thus the VWA domain of S5a is required for the proteasomal degradation of a subset of cytosolic proteins in muscle during fasting. It is noteworthy that the expression of S5aΔVWA was not sufficient to attenuate the reduction in fiber size during fasting (unpublished data), indicating that S5a's VWA domain is not required for the loss of contractile components in muscle, which are assembled within filaments and comprise the majority of muscle proteins (Beehler *et al.*, 2006; Ventadour and Attaix, 2006).

In light of the inhibitory role of S5aΔVWA and its higher capacity to bind polyubiquitin (Figure 5A), we sought to address directly the ubiquitin-binding regulation by the VWA. Recombinant S5a and S5aΔVWA were bacterially expressed and evaluated for their capacity to bind ubiquitin directly in an in vitro isothermal titration calorimetry (ITC) assay (Figure 5C). Our results reveal the higher affinity of S5aΔVWA for monoubiquitin (84 vs. 112 μM) and indicate that the enthalpy gain by the interaction with ubiquitin was lower in the S5aΔVWA mutant protein. Furthermore, the conformational transitions upon binding to ubiquitin were diminished, as indicated by the lower entropic penalty. As previously suggested (Isasa *et al.*, 2010), these results support the idea of the VWA domain forming an intramolecular interaction with the UIM domain within S5a. ITC evaluations of the affinity of S5a and S5aΔVWA mutant for higher-affinity monomeric ligand (hPlic-2 Ubl; Walters *et al.*, 2002) also indicated a similar increase in S5aΔVWA binding (see *Discussion*).

Consistent with our previous results (Figure 2), we noted a decrease in polyubiquitinated ATF4 levels upon reintroduction of S5a WT (but not S5a UIM mutant) in S5a kd cells (Figure 5D; input, long exposure). ATF4 levels were most responsive to expression of S5aΔVWA (Figure 5D; input), and S5a IP indicated preferred binding

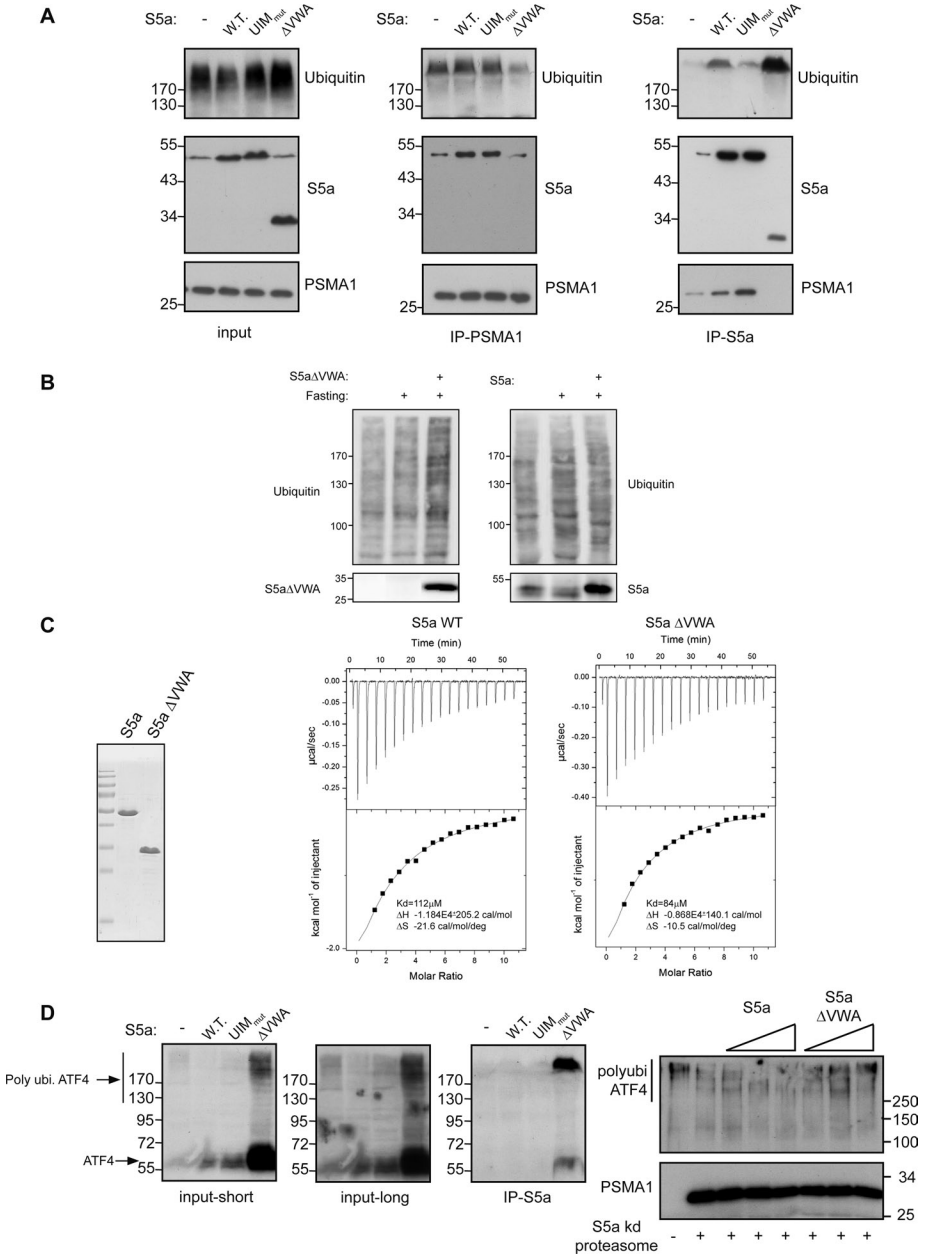

FIGURE 5: VWA regulates S5a binding to ubiquitin and Ubl. (A) S5a kd cells transfected with the indicated S5a constructs were Velcade treated and evaluated for polyubiquitin, PSMA1, and S5a content (input). S5a IP from the indicated transfections reveals the higher binding capacity of S5a ΔVWA for polyubiquitin and its inability to bind proteasomes (IP-S5a). Proteasome purifications (PSMA1 IP) show the lack of S5aΔVWA binding and reduced polyubiquitin content upon S5aΔVWA expression. (B) Tibialis anterior muscles were electroporated with the indicated expression vectors ,and 4 d later mice were deprived of food for 2 d as indicated. In fed mice, muscles were dissected 6 d after electroporation. Polyubiquitin and S5a contents of muscles were revealed by immunoblots. (C) Recombinant full-length or ΔVWA-domain S5a were bacterially expressed and purified (left; Coomassie). Affinities and thermodynamic parameters of interactions with ubiquitin were determined by ITC (right). (D) Left , S5a kd cells were transfected with Ha-ATF4 and the indicated S5a constructs were evaluated for their ATF4 content (input). S5a IP from the indicated transfections reveals the enhanced purification of polyubiquitinated ATF4 only upon S5a ΔVWA transfection (IP-S5a). Right, polyubiquitinated ATF4 was incubated with proteasomes (purified from S5a kd cells) and increasing amounts of the indicated recombinant S5a. Proteasomal and polyubiquitinated ATF4 contents were evaluated by the indicated immunoblots (PSMA1 and HA, respectively).

of S5aΔVWA to the polyubiquitinated species of ATF4 (Figure 5D; S5a IP). In spite of the expected reduction in proteasomal association with polyubiquitinated ATF4 in cells expressing S5aΔVWA, we could not address this point in vivo due to the drastic increase in ATF4 levels in these cells. Therefore we performed an in vitro degradation assay for polyubiquitinated ATF4, thus enabling us to have equal amounts of ATF4 substrate in all conditions examined. 26S proteasomes purified from S5a kd cells were incubated with polyubiquitinated ATF4 in the presence of increasing amounts of recombinant S5a or S5aΔVWA. As seen in Figure 5D, increasing S5a levels improved substrate degradation by the proteasome, whereas S5aΔVWA inhibited ATF4 degradation in a dose-dependent manner. These results are consistent with our in vivo results showing higher basal levels and lower proteasomal association of polyubiquitin upon S5aΔVWA expression (Figure 5A).

Multiubiquitination of S5a

Monoubiquitination events on Rpn10 have been described in yeast and *Drosophila* (Isasa *et al.*, 2010; Lipinszki *et al.*, 2012); however the multiubiquitination pattern in the two species was quite different. Recent reports also indicate ubiquitination of proteasomal ubiquitin receptors S5a and Rpn13 as a regulatory step in proteasomal substrate regulation (Besche *et al.*, 2014; Jacobson *et al.*, 2014). To evaluate the ubiquitination status of S5a in mammals, we immunoprecipitated S5a from proteasome-free and -bound fractions. As seen in Figure 6A, lower-mobility migration of S5a could be detected mainly in the proteasome-free fractions. The specific distribution of this event on S5a is readily seen when ectopic expression of green fluorescent protein (GFP)-S5a is performed and the reactivity of GFP-S5a is observed along the various fractions. As noted in Figure 6B (top), the lower-mobility GFP-S5a was restricted mainly to the LMW fractions, and this altered mobility could be abolished by incubation with a deubiquitinating enzyme (Figure 6B, bottom), indicating that ubiquitin is the post-translational modification responsible for the abnormal migration of S5a in the LMW fractions. Using the robustness of the ubiquitination events on ectopically expressed S5a enabled us to excise lower-mobility, Coomassie-stained bands from affinity-purified GFP-S5a and identify three in vivo monoubiquitination events on S5a, all occurring within the VWA domain (Figure 6C).

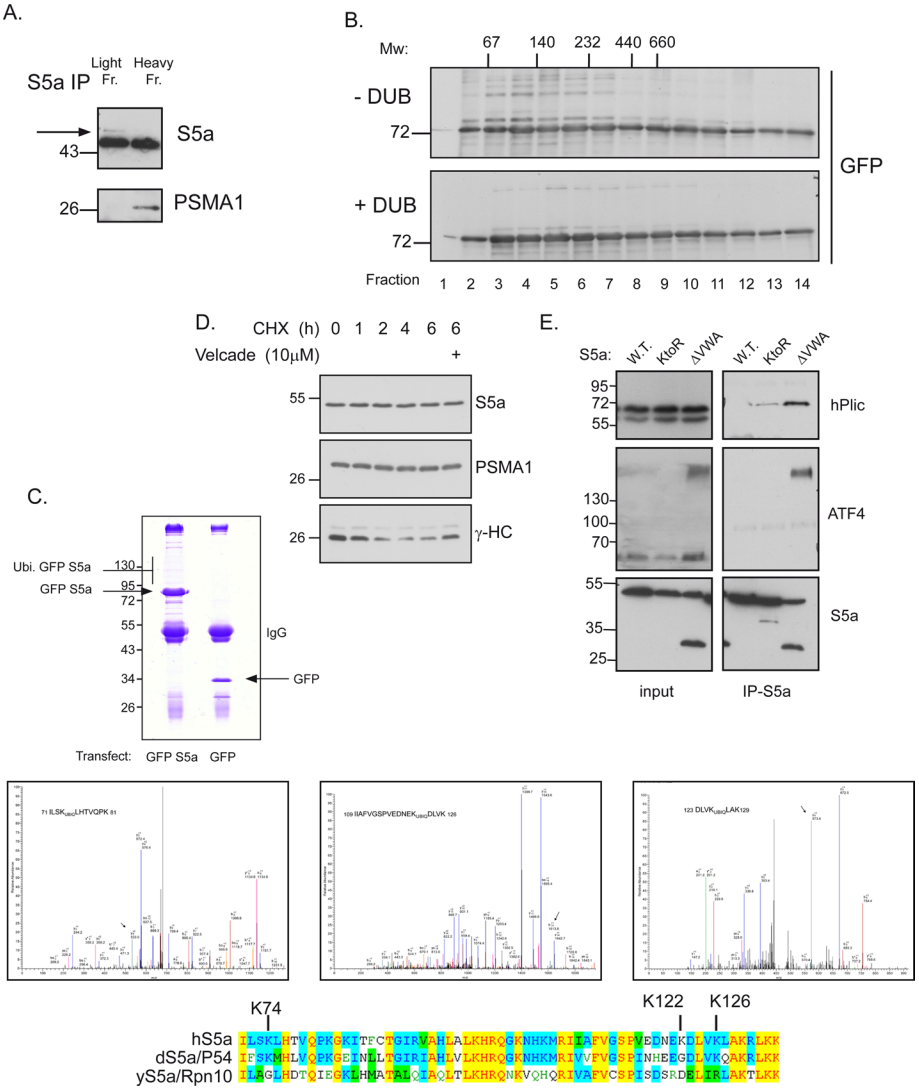

FIGURE 6: S5a multiubiquitination. (A) S5a IP was performed from LMW and HMW cellular fractions and immunoblotted for proteasomal (PSMA1) and S5a content. Note the appearance of a high-mobility S5a form in the LMW fractions (arrow). (B) Exogenous GFP-S5a was expressed in cells and distribution evaluated as in Figure 1A using a GFP immunoblot. Fractions along the gradient were presented as is or treated with the deubiquitinating enzyme USP2. Note the disappearance of the higher-mobility S5a-reactive bands upon USP2 addition. (C) Coomassie gel of GFP (control) and GFP-S5a, indicating the ubiquitinated bands excised for MS identification. MS spectra of the three identified sites and their aligned localization within the S5a VWA domain. (D) Cyclohexamide experiments revealing the long half-life of S5a and PSMA1 as opposed to the short half-life of an ERAD substrate (γV-CH1). (E) Cells transfected with HA-ATF4 and the indicated S5a were evaluated for their ATF4, S5a, and hPlic content (input). S5a IP from the indicated transfections was evaluated for copurification of hPlic and ATF4, revealing the partial ability of S5aKtoR mutant to enhance Ubl-dependent interaction with hPlic (IP-S5a).

These results are the first to characterize mammalian S5a ubiquitination sites and differ from those obtained by in vitro ubiquitination of Rpn10 (Isasa *et al.*, 2010) and the reported C-terminal end of *Drosophila* S5a/P54 (Lipinszki *et al.*, 2009). All three sites in S5a (K74, K122, and K126) are absent in the yeast Rpn10, and K122 is absent in *Drosophila* P54 (see Figure 6C for sequence alignment). Multiubiquitination of S5a does not seem to regulate protein stability, as S5a did not show a reduced half-life compared with other, long-lived proteasomal proteins (Figure 6D), in line with previous reports in *Drosophila* showing that ubiquitination events on P54 do not alter its own stability (Lipinszki *et al.*, 2009). We were able to confirm the previously reported reduced ubiquitin binding, but not proteasomal binding, of S5a-Ubi (a C-terminal fusion with ubiquitin reported to mimic Rpn10 ubiquitination and reduce ubiquitin binding; Isasa *et al.*, 2010; Supplemental Figure S3). Using a S5a mutant that is unable to undergo ubiquitination (KtoR mutant; unpublished data) did not reveal any changes in the ability to bind 26S proteasomes (Supplemental Figure S3). Use of this mutant can provide a tool to address the question of whether the enhanced capacity to bind polyubiquitinated ATF4 by S5aΔVWA is due to the lack of multiubiquitination or is part of an intrinsic property of VWA to regulate S5a ubiquitin binding. As seen in Figure 6E, S5a KtoR did not increase S5a binding to polyubiquitinated ATF4 as observed with S5aΔVWA. Because our previous results demonstrated S5a dependence for hPlic recruitment to the proteasome (Figure 3), we also addressed the ability of S5a KtoR and S5aΔVWA mutants to bind hPlic. Both mutations exhibited enhancement of hPlic binding, confirming the difference between Ubl and ubiquitin binding by S5a (our data; Walters *et al.*, 2002), as indicated by the ability of the KtoR mutant to enhance only Ubl binding. Thus we conclude that the multiubiquitination events found in the LMW fractions on S5a (Figure 3A) can contribute to the ability of the VWA domain to restrict S5a ability to bind Ubl only upon proteasomal integration.

DISCUSSION

Here we characterize the mammalian S5a with respect to its role in mediating substrate delivery to the proteasome. What are the consequences of S5a kd with respect to substrate degradation? What can we infer from these defects with regard to the role of S5a in substrate recognition and degradation? We show that polyubiquitinated ATF4 is a proteasomal substrate whose degradation and recruitment to the proteasome are highly dependent on S5a. This can be seen from the elevated levels and extended half-life of ATF4 in S5a kd cells (Figure 2). In spite of the higher levels of ATF4 in S5a kd cells, lower levels are found associated with the proteasome (Figure 2C). This situation is also found for polyubiquitin in general (higher levels and lower proteasomal association; Figure 2D). Ubl proteasomal association is also reduced in the S5a kd cells, as observed in the reduced hPlic and RAD23 Ubl-mediated proteasomal association (Figure 3). The consequence in both cases of ubiquitin and Ubl S5a-mediated proteasomal substrate delivery is the extended half-life of the specific substrates (ATF4 in the case of ubiquitin and the Sic1 substrate in the case of Ubl; Figures 2B and 3D, respectively).

Our findings show that S5a binding to hPlic occurs only in the presence of proteasomes even upon S5a overexpression (Figure 4),

and in S5a kd cells, residual levels of S5a are all fully retained in proteasomal fractions (Figure 1C). Furthermore, we were unable to identify S5a release from proteasomes upon in vitro degradation of a polyubiquitinated substrate, and we were unable to detect exchanges between proteasome-bound and -free S5a (unpublished data). These observations are also supported by findings showing S5a is present at stoichiometric amounts in 26S proteasome (Sakata *et al.*, 2012; Berko *et al.*, 2014). If the "normal life cycle" of S5a involved engagement and disembarkment with proteasomes, this would still be observed also in S5a kd cells, as cycling would still be expected to occur. These observations imply an additional role for proteasome-free S5a rather than its functioning as a proteasome-shuttling factor.

If S5a does not function as a proteasomal shuttle factor, how does its presence in the LMW fractions not perturb binding of polyubiquitinated substrates and Ubl-harboring proteins to S5a in the 26S proteasome? Deletion of the VWA domain that is required for proteasomal integration into the proteasome increased the binding of S5a to polyubiquitin chains both in vivo (cells and animals; Figure 5, A and B) and in vitro (Figure 5, C and D) and enhanced Ubl binding in vivo (Figure 6E). The VWA domain may reduce UIM functionality, and upon proteasomal binding to the VWA domain, this inhibition is relieved, thereby restricting S5a's ability to bind ubiquitin and Ubl selectively to the proteasome. Structural data on Rpn10/S5a VWA and UIM domains indicate a long, flexible linker between both domains of the protein (Riedinger *et al.*, 2010), one that may enable regulation of UIM functionality by the VWA domain. This mode of regulation has been observed for other catalytic proteasomal subunits (Hamazaki *et al.*, 2006; Yao *et al.*, 2006). Although our in vitro binding of S5aΔVWA to ubiquitin indicates only a slight increase in affinity (Figure 5C), this mutant binds to ubiquitin with different properties (Figure 5C; enthalpy and entropy values) and had a more profound effect on polyubiquitin chains (Figure 5, A and D). These differences cannot be simply explained by the higher affinity of S5a toward polyubiquitin as opposed to monoubiquitin, as similar differences in affinity were obtained with the higher-affinity Ubl domain of hPlic-2 (Supplemental Figure S2). Instead, it may be the repetition of the ligand (found only in polyubiquitin) that is required in order to unmask differences in binding properties of S5a versus S5aΔVWA, as the VWA may interfere only with one UIM binding site within S5a. Alternatively, and not mutually exclusive with the foregoing scenario, the existence of an inhibitory factor that associates with the non–proteasome-bound S5a VWA domain may explain the observed differences between in vivo and in vitro binding of the S5aΔVWA mutant toward ubiquitin and Ubl. Previous results indicating the role of S5a in p53 proteasomal degradation also demonstrated an inhibitory role for S5aΔVWA in p53 degradation in vivo (Sparks *et al.*, 2014), although in vitro experiments did not indicate such differences. This may be due to differences between in vivo experiments that would allow for inhibitory factor binding and in vitro experiments that would exclude such factors (Sparks *et al.*, 2014). Alternatively, the glutathione *S*-transferase (GST)–S5a fusion used in the in vitro experiments in Sparks *et al.* (2014) might alter S5a's ability to efficiently mediate substrate degradation (unpublished data) in addition to its effect on polyubiquitin binding by UIM domains (Sims and Cohen, 2009).

Previous reports regarding Rpn10/S5a multiubiquitination suggested a regulatory role in UIM functionality (Isasa *et al.*, 2010; Jacobson *et al.*, 2014). We found that multiubiquitination of S5a is present mainly in proteasome-free fractions and occurs only within the VWA domain (Figure 6). In this respect, the pattern of modification differs from the situation in yeast and *Drosophila* (Lipinszki *et al.*, 2009; Isasa *et al.*, 2010). As S5a binds ubiquitin via its UIM domains, it is important to acquire ubiquitinated peptides from in vivo events, as UIMs may alter ubiquitination pattern of the proteins acquired from an in vitro assay (Uchiki *et al.*, 2009), as evaluated in the case of the human S5a (Jacobson *et al.*, 2014). The mammalian and *Drosophila* S5a seem to share various traits; in both, there is no support for a role of S5a ubiquitination in regulating its own half-life (Figure 6D), and both ubiquitinated *Drosophila* and human forms are found in proteasome-free fractions (Lipinszki *et al.*, 2009). Multiubiquitination of S5a may play a role in limiting S5a's ability to bind ubiquitinated proteins, as ubiquitin fusion to S5a reduced its ability to bind polyubiquitin chains (Supplemental Figure S3). These observations were also confirmed for ubiquitin binding in yeast (Isasa *et al.*, 2010) and Ubl binding in *Drosophila* (Lipinszki *et al.*, 2009). In line with this hypothesis, we do not detect any effect of multiubiquitination on S5a proteasomal association (Supplemental Figure S3). To address whether the enhanced ubiquitin binding observed with S5aΔVWA is due to the elimination of multiubiquitination events occurring within the VWA domain or is intrinsic to the domain itself, we evaluated the ability of S5aKtoR to regulate ubiquitin and Ubl binding. Our results indicate that whereas the VWA KtoR mutant had reduced ubiquitin binding with respect to the WT S5a (S5a WT and KtoR mutant vs. S5a ΔVWA), its capacity to bind Ubl is enhanced (Figure 6E). Therefore multiubiquitination on VWA may further restrict S5a ability to bind Ubl domains. These differences between ubiquitin and Ubl are supported by our in vitro binding experiments showing higher affinity of S5a to hPlic-2 Ubl (Figure 5D and Supplemental Figure S2) and previous reports indicating a larger binding surface of S5a UIM with respect to Ubl (Walters *et al.*, 2002; Mueller and Feigon, 2003). Hence S5a ubiquitination can serve as a post-translational modification to restrict S5a from inhibiting substrate recruitment to the proteasome and may be used to regulate Ubl preference over ubiquitin binding to S5a.

A mechanism restricting the ability of nonintegrated proteasomal subunits to serve as an inhibitory factor would be important for any proteasomal subunit. The cell therefore restricts correct stoichiometric expression of the various proteasomal subunits by executing a transcriptional expression program (Mannhaupt *et al.*, 1999; Radhakrishnan *et al.*, 2010; Steffen *et al.*, 2010). Ubiquitin receptors would be especially susceptible to serve as inhibitory factors, and therefore it is important to maintain correct levels. S5a/Rpn10 is the only stoichiometric proteasomal ubiquitin receptor found in a non–proteasome-bound state (Hamazaki *et al.*, 2007) and would therefore require such mechanisms (VWA-domain inhibition and S5a multiubiquitination) to restrict its ability to bind ubiquitin/Ubl only upon proteasomal integration. Although nonintegrated S5a may not be able to compete for Ubl/ubiquitin chain binding, its cellular function remains to be determined. Several observations show that *rpn10*Δ phenotypes can be rescued by expression of a Rpn10UIM$_{mut}$ isoform (Fu *et al.*, 1998; Lin *et al.*, 2011), indicating an additional role for S5a/Rpn10. Further evaluation is required with respect to the additional roles of S5a/Rpn10, the mechanisms that regulate S5a ubiquitination, and its ability to sense the cellular UPS flux.

MATERIALS AND METHODS

Cell culture, transfection, lysis, and protein purification

HEK293 cells were cultured in complete medium composed of DMEM supplemented with 1% penicillin/streptomycin solution, 55 μM β-mercaptoethanol, 1% nonessential amino acids solution, and 10% heat-inactivated fetal bovine serum. Plasmids expressing GFP-S5a or S5a variants were constructed by amplifying the relevant amino acids of human S5a cDNA using pGEX.S5a plasmid

(a kind gift from Ivan Dikic, Goethe University, Frankfurt, Germany) as a template and ligating into the mammalian expression plasmid that omitted or harbored an N-terminal GFP tag (pEGFP; Clontech, Mountain View, CA) or a pIRES2-GFP vector. Rat ubiquitin was subcloned from a bacterial expression vector and constructed in-frame C-termini into the S5a mammalian expression vector. All single- and multiple-nucleotide mutations were performed using QuikChange Site Directed Mutagenesis or restriction enzyme substitutions. S5a VWA KtoR (substituting all 15 lysines within S5a VWA domain) was produced by GenScript (Piscataway, NJ), amplified, and constructed into the S5a vector. Knockdown of human S5a expression was obtained using a predesigned RNAi expression and control vector (human pGIPZ shRNA target gene set; Open Biosystems-Dharmacon, Lafayette, CO) to produce stable clones, and S5a rescue experiments on these cells were performed by expression of the indicated human S5a isoforms to obtain endogenous levels. Stable cell lines were selected using the puromycin-selectable marker by addition of 2 μg/ml puromycin to the medium of the transfected cells. Transient transfections were performed using the calcium-phosphate precipitation method or by PolyJet transfection reagent (SignaGen Laboratories, Rockville, MD) according to manufacturer's protocol.

Cell lysis was performed in TNH buffer (20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.9, 100 mM NaCl, 1% Triton X-100, 1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM dithiothreitol [DTT], 5 mM ATP, and protease inhibitors) and clarified at 20,000 × *g* for 10 min. Where indicated, 400 μl of lysate was layered over a 10–40% continuous glycerol gradient and centrifuged in a TH660 rotor (Sorvall; Thermo Fisher, Waltham, MA) for 4 h at 374,400 × *g*. Gradients were divided into 14 fractions. For direct proteasome purifications, cell lysates were immunopurified against PSMA1 or S5a antibody (Ab) as indicated, and beads were extensively washed with lysis buffer and subsequently eluted with Laemmli buffer; alternatively, soluble proteasomes were obtained by incubation with a PSMA1-eluting peptide (Stanhill *et al.*, 2006). For affinity purifications of proteasome-associated hPlic, proteasomes were first pelleted by a 60-min, 367,000 × *g* centrifugation, resuspended, and subsequently immunoprecipitated using a hPlic Ab. For lysates that are lacking proteasomal content, cell lysates were centrifuged for 60 min at 367,000 × *g*, and lysate supernatant was used. Where indicated, cells were treated with arsenite for the indicated time (0.5 mM; S7400; Sigma-Aldrich, St. Louis, MO) or Velcade as indicated (10 μM, 60 min). Conditions for all other protein misfolding agents are indicated in the figure legends.

Antibodies and Western blots

Psmd14 and P97 antisera were produced by immunizing rats and rabbits against full-length mouse Psmd14 and rat P97, respectively. GFP antiserum was produced by immunizing rabbits against full-length GFP. Antibodies and their sources were anti-Psma1 (ABR, Golden, CO) or monoclonal antibody 2-17 (a kind gift from Keiji Tanaka, Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan), anti-ubiquitin (Zymed-Invitrogen Waltham, MA), anti-Rpt1 (Biomol, Kelayres, PA), anti-Flag M2 (Sigma-Aldrich); anti-S5a (Abcam, Cambridge, UK; and a CPTC-PSMD4 hybridoma [52242. DSHB] obtained from the Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA), anti-hemagglutinin (HA; Covance, Princeton, NJ), anti-hPlic (Abcam), anti-P27kip1 (Abcam), anti-T7 tag (ICL, Portland, OR), and anti-tubulin (Sigma-Aldrich). Where shown, Western blotting quantifications were performed by ImageJ software (National Institutes of Health, Bethesda, MD) on at least three independent experiments.

Cyclohexamide assay

Cells were grown in DMEM and treated with 100 μg/ml cycloheximide, and lysates were prepared for SDS–PAGE analysis at the indicated time points as described previously (Isakov and Stanhill, 2011). For S5a-turnover evaluations, samples were analyzed by S5a, γV-CH1 (an endoplasmic reticulum-associated degradation (ERAD)–transfected substrate kindly provided by Linda Hendershot, St. Jude Children's Research Hospital, Memphis, TN; Okuda-Shimizu and Hendershot, 2007), and PSMA1 immunoblots (Figure 6) or HA, S5a, and PSMD14 immunoblots for ATF4 degradation assays (Figure 2).

Mass spectrometry

Gel bands containing S5a were reduced (2.8 mM DTT, 60°C for 30 min), modified (8.8 mM iodoacetamide in 100 mM ammonium bicarbonate, room temperature for 30 min), and digested in 10% acetonitrile and 10 mM ammonium bicarbonate with modified trypsin (Promega, Madison, WI) overnight at 37°C. The resulting tryptic peptides were separated by capillary reversed-phase liquid chromatography (50-min gradient on 0.075 × 200–mm fused silica capillaries; J&W, Folsom, CA) packed with Reprosil reversed-phase material (Dr. Maisch, Ammerbuch-Entringen, Germany) coupled to hybrid tandem mass spectrometer (Orbitrap; Thermo). Tandem mass spectrometry (MS) was performed in a positive mode using repetitively full MS scan, followed by collision-induced dissociation of the seven most dominant ions selected from the first MS scan. The MS data were analyzed using SEQUEST algorithm versus the human database, looking for modifications on S5a protein. Ubiquitination was identified as a mass addition of 114.0429 (signature diglycine generated by trypsin digest of conjugated ubiquitin) on lysine residues.

Proteasome activity and in vitro degradation assays

Recombinant full-length or ΔVWA-domain S5a was expressed as N-terminal GST fusion in pGEX-6. After isopropyl-β-D-thiogalactopyranoside induction and glutathione affinity purification, the proteins were eluted with a precision protease to yield only the untagged protein. The Sic1 degradation assay was performed as previously described (Saeki *et al.*, 2005) with a few modifications: proteasomes were purified from control or S5a kd cells as described but with 0.3% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate instead of Triton X-100. For UBL-dependent degradation assays, the UBL domain from RAD23UBL-DHFR (kindly provided by Andreas Matouschek, University of Texas, Austin, TX; Kraut and Matouschek, 2011) was inserted between the T7 and Sic1 to produce T7-UBL-PY-Sic1 and incubated with proteasomes from the various sources to evaluate substrate binding and degradation. Chemotryptic LLVY-AMC hydrolysis was performed as previously described (Isakov and Stanhill, 2011). For the in vitro ATF4 degradation assay, Ha-ATF4 was transfected into S5a kd cells, and ATF4 was subsequently lysed from Velcade- and *N*-ethylmaleimide–treated lysates. The affinity-purified ATF4 substrate was incubated in degradation buffer (50 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MgCl_2$, 10% glycerol, 1 mM DTT, and 2 mM ATP) for 30 min with soluble proteasomes purified from S5a kd cells in the presence of recombinant S5a as indicated. Reactions were terminated by boiling the sample in Laemmli buffer, and ATF4 degradation was evaluated by Ha immunoblots.

Isothermal titration calorimetry

All experiments were performed on an ITC200 microcalorimeter (MicroCal) at 25°C. The titrations were initiated with a 0.5-μl injection, followed by 18 2-μl injections with 3-min intervals of 3 mM

monoubiquitin (U6253; Sigma-Aldrich). Cells contained 22 μM indicated S5a in S buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 3 mM EDTA). Analysis was performed using the instrument software and removing initial injections from analysis. For Ubl-binding measurements (Supplemental Figure S3), cells contained 9.5 μM indicated S5a and 100 μM Ubl titrant.

In vivo gene transfection

Animal experiments were conducted according to the ethical guidelines of the institutional Animal Care and Use Committee. In vivo electroporation was performed in adult CD-1 male mice (24–27 g) by the injection of 20 μg of plasmid DNA into the tibialis anterior muscle and application of mild electric pulses (12 V, five pulses, 200-ms intervals; Cohen *et al.*, 2012). Muscle atrophy was induced by fasting, that is, food was removed from cages 5 d after electroporation for 48 h. Muscle homogenization was performed as described (Cohen *et al.*, 2012). Briefly, mouse tibialis anterior muscles were homogenized on ice in homogenization buffer (20 mM Tris-HCl, pH 7.2, 5 mM ethylene glycol tetraacetic acid, 100 mM KCl, 1% Triton X-100, and EDTA-free protease inhibitor cocktail) and spun at 6000 × *g* for 20 min to pellet nuclei, myofibrils, and unbroken tissue. The supernatant was analyzed by SDS–PAGE and immunoblotting.

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