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Heat shock protein gp96 regulates Toll-like receptor 9 proteolytic processing and conformational stability

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ABSTRACT

Nucleic acid-sensing Toll-like receptors (TLRs) initiate innate immune responses to foreign RNA and DNA, yet can detect and respond to host DNA. To avoid autoimmune pathologies, nucleic acid sensing TLRs are tightly regulated. TLR9 primarily resides in the endoplasmic reticulum, traffics to endosomes, is proteolytically processed and responds to DNA. The heat shock protein gp96 is one of several accessory proteins that regulate intracellular trafficking of TLR9. In the absence of gp96, TLR9 fails to exit the endoplasmic reticulum, and therefore gp96-deficient macrophages fail to respond to CpG DNA. However, absence of gp96 precludes studies on potential chaperoning functions of gp96 for TLR9. Here we demonstrate that pharmacologic interference with gp96 function inhibits TLR9 signaling. TLR9 remains associated with gp96 during intracellular trafficking, and gp96-specific inhibitors increase TLR9 sensitivity to proteolytic degradation. We propose that gp96 is critical for both TLR9 egress from the ER, and for protein conformational stability in the endosomal compartment. These studies highlight the importance of examining gp96-specific inhibitors for modulating TLR9 activation, and the treatment autoimmune diseases.

1. Introduction

Toll-like receptors (TLRs) participate in host defense [1], but must be tightly regulated because some detect molecular structures that are conserved between host and pathogen [2]. Nucleic acid sensing TLRs are regulated through localization, trafficking, and post-translational modifications [3–12]. Failures in regulation lead to inflammation and autoimmunity [13–19].

TLR9 senses DNA and is localized primarily to the endoplasmic reticulum (ER) [8,10]. A small pool of TLR9 constitutively traffics from the ER through the Golgi to the endolysosomal compartment [3]. Once in the endosomal compartment TLR9 is proteolytically processed in at least two locations [4,6,7,11,12]. These proteolytic events result in forms of TLR9 that positively [6,7,11,12], or negatively [4], regulate signaling.

The heat shock protein (Hsp) glycoprotein 96 (gp96) is one of s everal ER resident proteins that regulate TLR9 access to endosomes [20–29]. Macrophage-specific knockout of gp96 abolishes signaling through TLR9 because TLR9 remains in the ER [29]. Other Hsps regulate client protein conformation and ligand-binding, and Hsp inhibitors demonstrate therapeutic promise [30,31]. Previous studies have not addressed the possibility that gp96 regulates TLR9 stability and conformation.

In this study we have used a specific inhibitor to evaluate a potential role for gp96 on TLR9 stability. Our results show that specific inhibition of gp96 reduces TLR9 signaling, and that gp96 remains associated with TLR9 during traffic to the lysosomal compartment. When gp96 function is inhibited, TLR9 demonstrated an increased sensitivity to proteolytic degradation. Taken together, these data support a model in which gp96 plays a multi-faceted role in TLR9 biology, providing a rational basis for therapeutic targeting of gp96 in autoimmune disease.

2. Materials and methods

2.1. Reagents and plasmids

The following antibodies were used: HA for immunoprecipitation (ABM), HA for immunoblotting (Roche Applied Science), GFP (recognizes YFP) for immunoprecipitation (Invitrogen/Molecular Probes), GFP for immunoblotting (BD Clontech), LAMP-1, Rab 5 and Calnexin (BD-transduction laboratories), human gp96 (Santa

Abbreviations: ER, endoplasmic reticulum; GFP, green fluorescent protein; gp96, glycoprotein 96; HA, hemaglutinin; Hsp, heat shock protein; p80, 80 kDa TLR9 proteolytic fragment; TLR, Toll-like receptor; TNF-α, tumor necrosis factor alpha; UNC93B1, UNCoordinated-93 homolog B1.

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Cruz Biotechnology), HRP conjugated mouse, rat and rabbit secondary (Southern Biotech), HRP conjugated goat secondary (Jackson ImmunoResearch Labs), tubulin (eBioscience). The following reagents were used: geldanamycin (Sigma–Aldrich), poly I:C (Calbiochem), and CpG DNA 10104 5′-TCGTCGTTTCGTCGTTTTGTC GTT-3′ (Eurofins MWG Operon) Plasmids were prepared using Endo free kits (Qiagen Sciences). WS13, a gp96-specific Hsp90 inhibitor of the purine-scaffold class [32], was synthesized as previously described [33,34]. TLR9-HA was kindly provided by G. Barton (UC Berkeley).

2.2. Cell culture

Human Embryonic Kidney (HEK) 293 cells (ATCC #CRL-1573), RAW 264.7 cells (ATCC #TIB-71), and ØNX-Ampho cells (Orbigen) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2 mM L-glutamine, 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 10% low endotoxin FBS. Cells routinely tested negative for mycoplasma.

2.3. Retroviral transduction

Retroviral supernatants were generated using Lipofectamine 2000 (Invitrogen) transfected ØNX-Ampho cells. RAW 264.7 cells were infected (1811*g*, 32 °C, 90 min) with retroviral supernatants mixed with 8 μg/mL polybrene (Sigma). Following centrifugation, the media was changed and cells were incubated for 48 h at 37 °C.

2.4. Organelle fractionation

HEK293 cells stably expressing human TLR9 tagged at the N-terminus with hemaglutinin (HA-TLR9) [3] were incubated in homogenization buffer (HB: 0.25 M sucrose, 1 mM EDTA, 10 mM Tris, pH 7.4, and protease inhibitor cocktail) on ice for 15 min, homogenized, and passed through a 25 G needle. Combined post-nuclear supernatants (two times 900*g* for 10 min at 4 °C) were layered on Percoll in HB (density 1.05 g ml^{-1}) and centrifuged for 60 min at 34,000*g* in a 50Ti rotor using a Beckman L-8 M ultracentrifuge. Fractions (500 μl each) were collected from the top using an AutoDensi-Flow IIC gradient unloader (Haake Buchler). Laemmli reduced sample buffer (final concentrations 62.5 mM Tris pH 6.8, 12.5% glycerol, 1% SDS, 0.005% bromophenol blue, 1.7% β-mercaptoethanol) was added and proteins were resolved by SDS–PAGE. Nitrocellulose membranes were immunoblotted for the indicated proteins.

2.5. Co-immunoprecipitation

HEK293 cells were pretreated as described then lysed (Lysis buffer: 137 mm NaCl, 20 mM Tris pH 7.4, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100, protease inhibitor cocktail (Roche) and 100 mM phenylmethylsulphonyl fluoride) and incubated with anti-TLR9 overnight or anti-HA or anti-GFP for two hours before adding protein A/G Sepharose beads and incubating for an additional one hour. After washing three times in lysis buffer, Laemmli reduced sample buffer was added, samples were boiled, and proteins were resolved by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were immunoblotted as described. For sequential immunoblotting, membranes were stripped with Restore (Pierce).

2.6. Cytokine ELISA

The mouse TNF-α ELISA MAX Set (BioLegend) was used according to manufacturer's instructions.

2.7. In vitro proteolysis assay

RAW 264.7 cells retrovirally transduced with TLR9-HA were treated as indicated, lysed in Lysis buffer and incubated at 4 or 24 °C for the indicated times. Reactions were terminated with the addition of sample buffer. Samples were boiled and proteins were resolved by SDS–PAGE, transfered to nitrocellulose, and immunoblotted as indicated.

2.8. Densitometric analysis

Quantification of protein bands was performed as previously described [3].

2.9. Statistical Analysis

Student's two-tailed *t* test was used for comparing drug treatments.

3. Results

3.1. Disruption of gp96 function inhibits TLR9 response to CpG DNA

To address whether gp96 had additional regulatory functions on TLR9 besides regulating ER exit, we treated cells with a selective gp96 inhibitor, WS13 (EC_{50}^{Hsp90} = 33 μM and EC_{50}^{gp96} = 0.16 μM) [35]. Treatment with this inhibitor will block all gp96 function in the cells, but since TLR9 does not exit the ER in the absence of functional gp96, our studies will evaluate the effect of loss of gp96 function on TLR9 outside of the ER. The TLR9 ligand CpG DNA induced TNF-α production from mouse macrophages (RAW 264.7), and WS13 inhibited this response in a concentration dependent manner (Fig. 1A). Treatment with vehicle alone did not inhibit signaling (Fig. 1A). Importantly, WS13 did not inhibit TNF-α production by another nucleic acid-sensing TLR (TLR3), or by a surface expressed TLR (TLR4) (Fig. 1B and C). These results indicate that gp96 function is required for TLR9-dependent responses to CpG DNA.

3.2. gp96 is localized with TLR9 after exit from the ER

We next asked whether gp96 association with TLR9 is transient or long-term. HEK293 cells stably expressing N-terminally hemaglutinin-tagged human TLR9 (HA-TLR9) were treated with cycloheximide for various times, and the interaction between TLR9 and gp96 was determined by co-immunoprecipitation (Fig. 2A). There was a slight decrease in the amount of TLR9 immunoprecipitated with one-hour cycloheximide treatment, but the amount of gp96 co-immunoprecipitated with TLR9 remained constant during the eight-hour treatment (Fig 2A). The cycloheximide had no effect on the total amount of gp96 protein (Supplementary Fig. S1). Therefore, we conclude that gp96 remained associated throughout the lifetime of the TLR9 protein.

To determine whether TLR9 and gp96 traffic to the same compartment after TLR9 exits the ER, we performed immunoblot analysis on fractions from HEK293 cells stably expressing N-terminally hemaglutinin-tagged human TLR9. Organelle fractionation resolved early endosomes (Rab5), ER (calnexin) and lysosomes (LAMP-1) (Fig. 2B). Note that some Rab5 and LAMP-1 overlapped with calnexin-positive ER fractions but $Rab5^{+}LAMP\text{-}1^{-}$ and $Rab5^{-}LAMP\text{-}1^{+}$ fractions were distinct. Approximately 6–13% of TLR9 and 37.1% of gp96 was detected in $LAMP\text{-}1^{+}Rab5^{-}$ fractions. To confirm that TLR9 and gp96 directly interacted in the lysosomal fractions, the $LAMP1^{+}Rab5^{-}$ fractions were combined and immunoprecipitated for either TLR9 or gp96. Both TLR9 and gp96 were

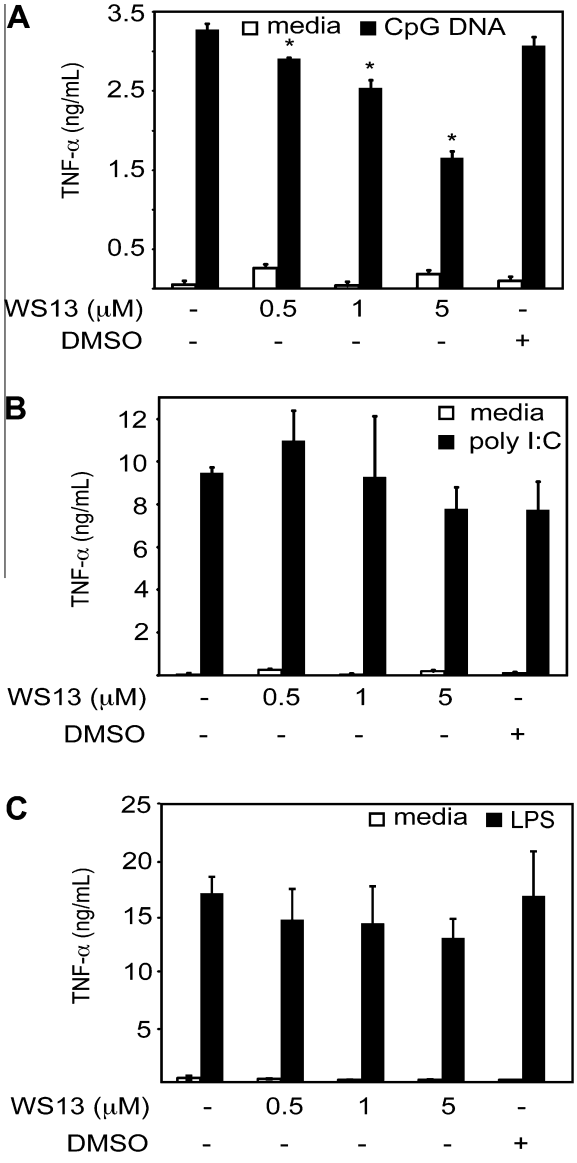

Fig. 1. Inhibition of gp96 reduces CpG DNA-induced responses. (A) RAW 264.7 cells were pretreated for 2.5 h with the indicated concentrations of WS13, or with DMSO, and stimulated with 0.5 μM CpG DNA for 24 h. (B) As in (A) except cells were stimulated with 5 μg/ml poly I:C for 24 h. (C) As in (A) except cells were stimulated with 100 ng/mL LPS for 24 h. Secreted TNF-α was determined by ELISA. $^{*}P < 0.005$. Representative of two independent experiments.

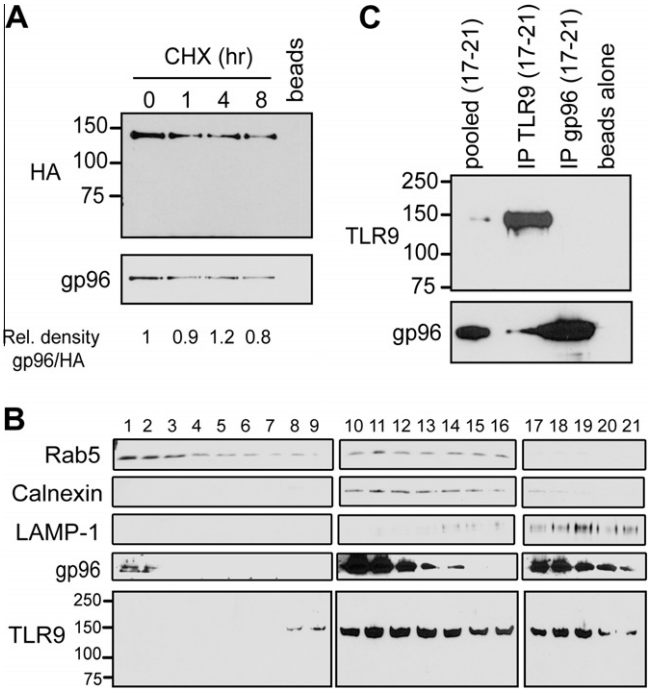

Fig. 2. TLR9 and gp96 traffic to the lysosome. (A) HEK293 cells stably expressing N-terminally HA-tagged human TLR9 (HA-TLR9) were pretreated with 10 μg/ml cycloheximide (CHX) for the indicated times prior to cell lysis and HA immunoprecipitation followed by HA and gp96 immunoblotting. Relative density for gp96 co-immunoprecipitation with TLR9 is indicated for each treatment condition. Representative of two independent experiments. (B) Sub-cellular fractions from HEK293 cells stably expressing N-terminally HA-tagged human TLR9 (HA-TLR9) were resolved by SDS–PAGE, and immunoblotted for early endosomes (Rab5), ER (calnexin), Lysosomes (LAMP-1), HA (TLR9), and gp96. Representative of three independent experiments. (C) TLR9 and gp96 were immunoprecipitated from combined fractions 17–20 from (B). Precipitates were resolved and immunoblotted for HA, and gp96. Combined fractions without immunoprecipitation, and beads alone controls are also shown.

immunoprecipitated from the pooled LAMP1^{+}Rab5^{-} fractions, and were individually immunoprecipitated from pooled fractions. Furthermore, gp96 was detected in the TLR9 immunoprecipitate, confirming that gp96 remained associated with TLR9 in lysosomes (Fig. 2C). There are likely additional gp96 client proteins in the lysosomal compartment since a significantly greater fraction of gp96 was present in this compartment than TLR9; however, we may have failed to detect some TLR9 since a proteolytic processing event occurs in endosomes that removes leucine rich repeats 1–14. We were unable to determine the percent of proteolytically processed TLR9 in this assay for two reasons: (1) in HEK293 cells, TLR9 was poorly proteolytically processed in the absence of co-transfected UNC93B1 (J. Brooks and C. Leifer, unpublished observation) and (2) the TLR9 in this experiment was N-terminally tagged and the anti-tag antibody would not detect the proteolytically cleaved form. Together, these results confirm a prolonged association between TLR9 and gp96 outside of the ER and prompted us to examine the possibility that gp96 stabilized or protected TLR9 from degradation in the endosomal compartment.

3.3. Inhibition of gp96 function enhances TLR9 proteolytic sensitivity

Since TLR9 remained associated with gp96, and pharmacologic inhibition of gp96 inhibited TLR9 signaling, we next asked whether inhibition of gp96 function induced degradation of TLR9. We used two different inhibitors, geldanamycin, an inhibitor of both gp96 and Hsp90, and WS13, a specific gp96 inhibitor. To determine whether either drug disrupted the interaction between TLR9 and gp96, we used HEK293 cells stably expressing wild-type human TLR9 C-terminally tagged with YFP (9-YFP). TLR9 and gp96 were co-immunoprecipitated from untreated, but not geldanamycin treated cells (Fig. 3A). However, WS13 treatment did not change the amount of gp96 co-immunoprecipitated with TLR9 even with higher concentrations of drug (Fig 3A). These data are consistent with previous studies showing that WS13 does not disrupt gp96 association with client proteins, but geldanamycin does [35]. We conclude that the two drugs, although both inhibitors of gp96, have different mechanisms of action and we could therefore use them to evaluate whether gp96 association, or function, had an effect on TLR9 stability.

We next used the more relevant system, RAW 264.7 macrophages, to address the result of inhibition of gp96 function on TLR9 protein. RAW264.7 cells were retrovirally transduced with C-terminally HA-tagged mouse TLR9. Both full-length TLR9 and the 80 kDa proteolytic fragment were detected in whole cells

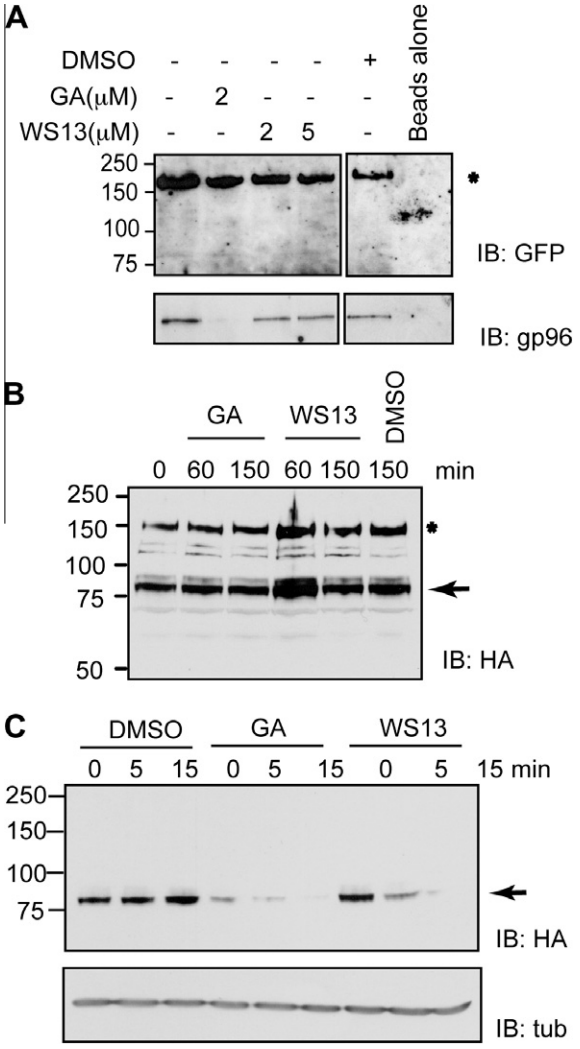

Fig. 3. Inhibition of gp96 increases TLR9 sensitivity to proteolytic digestion. (A) HEK293 cells stably expressing human TLR9-YFP were treated with DMSO or the indicated concentrations of geldanamycin (GA) or WS13 for 1 h. Cells were lysed, immunoprecipitated for GFP, or with no antibody (beads alone), and immunoblotted (IB) for GFP and gp96. Representative of two independent experiments. (B) RAW 264.7 cells were transduced with C-terminally HA-tagged mouse TLR9 (TLR9-HA) and treated with 2 μM GA, 2 μM WS13, or DMSO control for the indicated times. Whole cell lysates (WCL) were assayed by immunoblotting (IB) for HA. Representative of two independent experiments. (C) RAW 264.7 cells were transduced and treated as in (B). Lysates were prepared as for immunoprecipitation, incubated at 24 °C for the indicated times (0 is kept at 4 °C), and assayed by immunoblotting (IB) for HA and tubulin (tub). Representative of two independent experiments.* Full length TLR9, ← proteolytically cleaved TLR9.

lysates. Treatment with either geldanamycin or WS13 for up to two and ½ hours did not alter the abundance of either form as detected in lysates prepared directly in Laemmli sample buffer, and boiled immediately (Fig. 3B).

To test whether inhibition of gp96 had an effect on TLR9 proteolytic sensitivity, we performed an in vitro proteolysis assay. Retrovirally transduced RAW 264.7 cells expressing TLR9-HA were treated with geldanamycin or WS13, and lysates (in traditional triton X-100 containing buffer with a protease inhibitor cocktail) were warmed to 24 °C for five or 15 min, or kept at 4 °C (0 min). In lysates from DMSO treated cells, the abundance of a control protein, tubulin, was not reduced during the 15-min incubation at 24 °C (Fig. 3C). Neither geldanamycin, nor WS13, treatment altered abundance of tubulin. Without drug treatment, we only observed the 80 kDa proteolytic form of TLR9 in these lysates. These data indicate that upon lysis of macrophages, TLR9 becomes exposed to, and processed by, endosomal proteases such as cathepsins [6,12], whose inhibitors are not in the protease inhibitor cocktail. However, upon warming the lysates to room temperature, cells pretreated with geldanamycin, or WS13, had reduced abundance of TLR9. Loss of TLR9 protein was a time-dependent (Fig. 3C). From these studies we conclude that loss of gp96 function leads to increased sensitivity of TLR9 to proteolysis. Since the two inhibitors have different mechanisms of action, we interpret these data to mean that gp96 function, not just association, is required to protect TLR9 from proteolysis and maintain TLR9 conformational stability.

4. Discussion

Mechanisms to block signaling through nucleic acid-sensing TLRs that recognize shared structures between pathogen and host have therapeutic potential in treating autoimmune disease. In this study, we show that signaling through TLR9 is reduced by specific pharmacologic inhibition of the endoplasmic reticulum chaperone gp96. TLR9 remained associated with gp96 following exit from the ER, and the specific gp96 inhibitor, or a general Hsp90 class inhibitor, promoted TLR9 degradation. We propose that inhibition of gp96 alters the conformation of endosomal TLR9, and since this pool initiates signaling, gp96 inhibition reduces TLR9 signaling. Therefore, specific gp96 inhibitors should be investigated further for autoimmune therapy.

Full chaperoning activity of gp96 is required for TLR9 signaling. Crystallographic and biochemical investigations demonstrate that geldanamycin preferentially interacts with Hsp90 in an apo, or open-conformation, that is unfavorable for client protein binding [36–38], while WS13 preferentially associates with an ATP-bound conformation of the chaperone and stabilizes the client protein/Hsp interaction [32]. Thus, while recognizing similar Hsp90 species, WS13 and geldanamycin induce distinct conformations in Hsp90 and/or gp96 upon binding, leading to client protein trapping or release, respectively. Since both drugs inhibit TLR9 function and induce proteolytic sensitivity, we conclude that fully functional gp96, that can associate and dissociate from TLR9, is required for TLR9 conformational stability and function.

Data presented here support a role for gp96 in maintaining conformational stability of the TLR9 ecto-domain. Treatment with geldanamycin or WS13 increased TLR9 sensitivity to proteolytic processing without altering abundance (Fig. 3). In our hands we observe much more of the 80 kDa mature proteolytic fragment in macrophage lysates than full-length TLR9, making detection of the full-length protein difficult (Fig. 3C). Previous studies demonstrated that TLR9 undergoes a major conformational change upon ligand binding [39]. Our data are consistent with gp96 maintaining TLR9 in basal conformation prior to ligand binding. Without gp96, TLR9 likely unfolds, increasing its sensitivity to proteases when cells are lysed. Therefore, we believe that gp96 regulates TLR9 function by maintaining protein stability in a ligand-accessible conformation (Supplementary Fig. S2). gp96 is itself destabilized at pH $\leqslant$ 5 [40], and therefore, gp96 would maintain the ligand-binding conformation of TLR9 in early endosomes, but upon reaching a pH less than 5, gp96 would dissociate and TLR9 conformation would change.

In summary, we propose a new model whereby gp96 regulates TLR9, both in the ER to ensure full maturation, and after ER exit to maintain conformation. In the absence of gp96, TLR9 fails to exit the ER and does not signal. In the presence of functional gp96, TLR9 traffics from the ER, and remains associated with gp96 over the half-life of TLR9, even in the lysosomal compartment. Inhibition of gp96 function at this point enhances TLR9 sensitivity to proteolysis indicating a change in TLR9 conformation. We propose

that WS13-mediated inhibition of gp96 may improve inflammatory symptoms in diseases in which TLR9 plays a role, such as systemic lupus erythematosus or rheumatoid arthritis.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.04.083.

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