Endotoxin Conditioning Induces VCP/p97-mediated and Inducible Nitric-oxide Synthase-dependent Tyr\(^{284}\) Nitration in Protein Phosphatase 2A\(^{\text{XY}}\)

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Takashi Ohama and David L. Brautigan

From the Center for Cell Signaling and Department of Microbiology, University of Virginia School of Medicine, Charlottesville, Virginia 22908

Endotoxins activate Toll-like receptors and reprogram cells to be refractory to secondary exposure. Here we found that activation of different Toll-like receptors elicited a time- and dose-dependent increase in the levels of the protein phosphatase 2A catalytic subunit (PP2Ac) but not its partner A subunit. We purified the lipopolysaccharide-induced form of PP2A by chromatography plus immunoprecipitation and used mass spectrometry to identify VCP/p97 as a novel partner for PP2Ac. Endogenous VCP/p97 and PP2Ac were co-immunoprecipitated from primary murine macrophages and human lymphocytes. GST-VCP/p97 bound purified PP2A in pulldown assays, showing direct protein-protein interaction. Endotoxin conditioning of macrophages induced formation of 3-nitrotyrosine in the PP2Ac associated with VCP/p97, a response severely reduced in macrophages from iNOS knock-out mice. The reaction of purified PP2A with peroxynitrite dissociated the A subunit, and macrophages from iNOS knock-out mice. The reaction of purified PP2A with peroxynitrite dissociated the A subunit, and macrophages from iNOS knock-out mice.

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out the scaffold A subunit. One such example is direct binding of PP2Ac to α4 (yeast Tap42), which displaces the A subunit (12), but this involves a small fraction of PP2Ac in cells. Mechanisms to regulate the distribution of PP2Ac among various complexes are not understood.

Post-translational modification of PP2Ac such as phosphorylation or methyl-esterification regulates PP2A activity and holoenzyme composition (13). Tyrosine phosphorylation of purified PP2Ac by kinases occurs on Tyr^{307} and inactivates PP2Ac (14). PP2Ac tyrosine phosphorylation in cells is induced by v-Src expression or epidermal growth factor addition (15). Anti-Tyr(P)^{307} phosphosite antibodies from multiple commercial sources have been used to show PP2Ac phosphorylation in response to various stimuli. On the other hand, mass spectrometry shotgun Tyr(P) proteomics identified phosphorylation of Tyr^{284} but not Tyr^{307} in PP2Ac in v-Src transformed fibroblasts (16). More recently, nitration of unidentified Tyr in PP2Ac was correlated with increased PP2Ac activity in endothelial cells treated with inflammatory stimulus or peroxynitrite (17). Nitration reduced Tyr^{307} phosphorylation of PP2Ac, suggesting that this residue is either phosphorylated or nitratated. In iNOS KO cells there was neither Tyr nitration nor activation of PP2Ac, showing that the process probably involved peroxynitrite generation from nitric oxide (17).

Here, we discovered that endotoxin conditioning with different TLR ligands produced a time- and concentration-dependent elevation of PP2Ac levels without a parallel increase in A subunit. We purified by chromatography the endotoxin-induced surplus PP2Ac and co-immunoprecipitated VCP (valosin-containing protein)/p97, a highly conserved hexameric AAA ATPase involved in a variety of cellular functions, including ubiquitin-mediated proteosomal degradation (18). Preconditioning induced iNOS-dependent Tyr nitration in PP2Ac associated with VCP/p97. Peroxynitrite reaction of PP2A AC dimer caused dissociation, and the nitration was mapped by mass spectrometry to Tyr^{284}. Transient expression of PP2Ac wild type and Y284F in cells treated with peroxynitrite showed differences in Tyr nitration and A subunit co-precipitation. Expression of VCP/p97 or PP2Ac elevated DUSP1 levels and limited the activation of p38 MAPK and the release of TNF-α, mimicking endotoxin conditioning. Our results support a novel mechanism for adaptive tolerance, involving VCP/p97 recruitment to PP2A to promote Tyr^{284} nitration, A subunit dissociation, and elevation of DUSP1 to make cells refractory to stimulation.

**EXPERIMENTAL PROCEDURES**

**Animal and Cell Culture**—RAW264.7 cells and human peripheral blood mononuclear cells (PBMCs) were obtained from ATCC and Virginia Blood Services (Richmond, VA.), respectively, and grown in RPMI 1640 supplemented with 10% fetal bovine serum and 1× anti-biotic/anti-myotic (Invitrogen). HEK293T cells were obtained from ATCC and grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. Mouse bone marrow-derived macrophages (BMDMs) were isolated and grown as previously described (19). iNOS knock-out mouse was provided by Dr. Victor Laubach (University of Virginia).

**Immunoblotting**—The cells were lysed in a buffer containing 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.2% SDS, 0.5% Nonidet P-40, 1 mM Na_{3}VO_{4}, 1 μM Microcystin-LR, and Roche Complete protease inhibitor mixture. The proteins were separated by SDS-PAGE and transferred onto nitrocellulose membrane (Bio-Rad). The membranes were blocked with 3% skim milk and treated with antibodies described and quantified by LI-COR Odyssey infrared scanner and software. Actin or glyceraldehyde-3-phosphate dehydrogenase was immunoblotted as a loading control.

**DEAE Chromatography**—RAW 264.7 cells were treated with or without lipopolysaccharide (LPS; 0.1 μg/ml) for 16 h. The cells were suspended in buffer (25 mM Tris-HCl, pH 7.5, 1 mM EGTA, 0.1% 2-mercaptoethanol, and Roche Complete protease inhibitor mixture) and passed through a 28 G 1/2 1-cc insulin syringe. 1 mg of total protein of the supernatants was loaded onto a DEAE MEM SEP HP1000 column (Millipore) pre-equilibrated in buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EGTA, and 0.1% 2-mercaptoethanol). Fractions were collected every minute (flow rate, 0.65 ml/min). The column was washed with buffer A for 15 min, followed by a linear gradient to buffer B (Buffer A with 500 mM NaCl) over 40 min. The fractions were precipitated with 10% trichloroacetic acid and 0.1% deoxycholate. The precipitates were redissolved in 1× SDS sample buffer, resolved on SDS-polyacrylamide gels, and immunoblotted. For PP2Ac and PP2A A subunits, band density of the individual fractions was expressed as a percentage of the total band density in all of the fractions.

**Gel Permeation Chromatography**—Fractions 40–45 from DEAE chromatography were pooled, concentrated, and injected onto a Superose 12 (10 × 300 mm) column (GE Healthcare) that was eluted at 0.5 ml/min at room temperature in a buffer containing 25 mM Tris-HCl (pH 8.0), and 150 mM NaCl. The Superose 12 column calibration with 10 protein standards and blue dextran is posted on-line by the European Molecular Biology Laboratory. The fractions were collected every 2 min and precipitated with 10% trichloroacetic acid and 0.1% deoxycholate. The precipitates were redissolved in 1× SDS sample buffer, resolved on 10% SDS-polyacrylamide gels, and immunoblotted.

PP2A AC dimer (Millipore, 2 unit) was treated with 110 pmol of peroxynitrite or degraded peroxynitrite in 50 μl of reaction buffer (100 mM Tris-HCl, pH 7.4, 25 mM sodium bicarbonate, and 0.5 mM EGTA) while vortex mixing, because the half-life of peroxynitrite is very short. PP2A was injected onto a Superose 12, and fractions were collected every 1 min. PP2Ac and A subunit were detected by immunoblotting.

**Immunoprecipitation**—The antibodies were bound to protein G-agarose beads and covalently cross-linked by dimethyl pimelimidate treatment. The cells were suspended in buffer consisting of 50 mM MOPS-NaOH (pH 7.4), 125 mM NaCl, 1 mM EGTA, 0.1% 2-mercaptoethanol, and Roche Complete protease inhibitor and passed through 28 G 1/2 syringe. For some samples, 1% of Nonidet P-40 was added to cell suspensions and sonicated four times for 5 s each. The supernatants were incubated with antibody-coupled beads. Normal IgG coupled to beads was used as control.
FIGURE 1. Conditioning with LPS induces novel PP2A holoenzyme. A, RAW264.7 cells were conditioned with or without LPS (0.1 μg/ml) for 16 h. The TNF-α level in the medium was measured by ELISA after 4 h of secondary LPS challenge. The data are the means ± S.E. (n = 4). B, cells were treated with LPS (0.1 μg/ml) for increasing time, and the levels of PP2A catalytic (PP2Ac) and PP2A A subunits (PP2A A) were quantified by immunoblotting, normalized to t = 0. Representative images from three independent experiments are shown. C, cells were treated with increasing concentrations of various endotoxins, LPS, CpG-DNA, or lipoteichoic acid (LTA) for 16 h. The levels of PP2A subunits were determined by immunoblotting. Representative images from four independent experiments are shown. D, dose-dependent increases in PP2Ac from quantified data. (means ± S.E., n = 4). E-H, cells were cultured with or without LPS (0.1 μg/ml) for 16 h (defined as conditioning). The extract proteins were applied to DEAE ion exchange and eluted with linear gradient from 0 to 0.5 M NaCl (dotted line). Each fraction was analyzed by immunoblotting for PP2A subunits. Representative images from two independent experiments for control (E) and conditioned (F) cells are shown. Immunoblotting of individual fractions for PP2Ac (G) and PP2A A subunit (H) was used to compare control (black line) and conditioned (red line) cells. The proteins were quantified as the percentages of the sum in all the fractions. The major peaks of PP2Ac are labeled P1–P4.

Plasmid and siRNA Transfection—RAW264.7 cells were transfected using Amaxa nucleofector system following Amaxa guidelines, cultured for 24 h, and used for experiments.

Mass Spectrometry—To detect PP2Ac binding partners, fractions 40–45 from DEAE chromatography were collected and dialyzed with buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 0.1% 2-mercaptoethanol. The sample was incubated overnight at 4 °C with anti-PP2Ac IgG cross-linked on protein G beads. After four washes with dialysis buffer, elution buffer (50 mM Tris-HCl, pH 6.8, 0.4% SDS, 6% 2-mercaptoethanol, and 2% glycerol) was added to the beads and left overnight at 4 °C. The eluted proteins were analyzed by liquid chromatography/MS as previously described (20).

For tyrosine nitration site detection, purified PP2A pulled down on MCLR beads was treated with peroxynitrite. The sample was eluted with SDS and resolved by SDS-PAGE, and the band of PP2Ac was digested with trypsin or Asp-N. The data were analyzed by data base searching using the Sequest search algorithm against the α and β isoforms of PPP2AC. Parent tolerance was 8 ppm, and fragment tolerance was 1 Da. The potential matches were first filtered by xcorr (+1 > 1.8, +2 > 2.3, +3 > 2.7, +4 > 3.7). Any peptides that passed these initial filters as being modified were then manually confirmed.

GST Pulldown Assay—Bacterially expressed GST-p97 or GST alone was bound with glutathione-agarose beads. PP2A AC dimer from human red blood cell (Millipore) was incubated with GST-p97 beads or GST beads in an assay buffer (50 mM MOPS-NaOH, pH 7.4, 125 mM NaCl, 1 mM EGTA, 0.1% 2-mercaptoethanol, and Roche Complete protease inhibitor mixture) overnight at 4 °C. The precipitates were resolved by SDS-PAGE and analyzed by immunoblotting.

ELISA—RAW264.7 cells were conditioned with LPS (0.1 μg/ml) or medium for 16 h and washed with phosphate-buffered saline before stimulation. For overexpression experiments, RAW264.7 cells were plated 24 h before stimulation and stimulated with LPS (0.1 μg/ml) for 4 h, and the culture medium was analyzed by Mouse TNF-α ELISA kit (R & D Systems) according to the manufacturer’s protocol.

Statistical Analysis—The results are expressed as the means ± S.E. Comparisons between the groups were performed by one-way analysis of variance, followed by Student-Newman-Keuls test. For all of the analyses, a probability value of p < 0.05 was considered to indicate statistical significance.

RESULTS

Endotoxin Conditioning Increases PP2Ac Levels without Increase in PP2A A Subunit—LPS, a membrane glycolipid of Gram-negative bacteria and a well known potent activator of TLR-4, was added for up to 16 h to condition mouse RAW264.7 cells. LPS-induced tolerance was demonstrated by the significant decrease in TNF-α release upon secondary exposure to LPS (Fig. 1A). Exposure to LPS induced a time-dependent, statistically significant 30% increase in the levels of PP2Ac protein without a parallel increase in the scaffolding A subunit of PP2A (Fig. 1B). Furthermore, we observed a dose-dependent significant increase in the levels of PP2Ac protein in cells treated with different endotoxin components: LPS (TLR4 agonist), CpG DNA (TLR9 agonist), or lipoteichoic acid (TLR2/6 agonist) (Fig. 1, C and D). In contrast, the levels of PP2A A subunit, B56y3 regulatory subunit, and alternate phosphatase subunit α4 did not increase in response to any these treatments, supporting the hypothesis that a nontypical form of PP2A accumu-
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FIGURE 2. PP2A associates with VCP/p97. A, PP2Ac was immunoprecipitated (IP) from fractions 40–45 of DEAE chromatography, and VCP/p97 was identified by liquid chromatography/MS. The black bars indicate positions of 23 peptides in the sequence that provided 35% coverage. B, reciprocal co-immunoprecipitation of the endogenous VCP/p97 and PP2Ac from RAW264.7 cells. Normal IgG was used as control (Cont). Representative images from two independent experiments are shown. C, human PBMCs were conditioned with LPS (10 ng/ml) for 16 h. The levels of PP2A and VCP/p97 in whole cell (WC) extracts were analyzed by immunoblotting (left panel). VCP/p97 was immunoprecipitated, and PP2A and PP2A A subunit association was analyzed by immunoblotting (right panel). Representative images from three independent experiments are shown. D, Superose 12 gel chromatography of fractions 40–45 from DEAE chromotography from LPS-conditioned RAW264.7 cells. The proteins in individual fractions were detected by immunoblotting with indicated antibodies. Representative images from two independent experiments are shown. E, binding of purified PP2A to immobilized GST-VCP/p97. Top panel, anti-GST immunoblot showing GST and GST-VCP/p97 fusion protein. Bottom two panels, bound PP2A and PP2A A subunits were detected by immunoblotting. Representative images from two independent experiments are shown.

To analyze PP2A holoenzymes in control versus LPS-conditioned cells, we examined the distribution of PP2Ac using DEAE ion exchange chromatography and immunoblotting. The endogenous PP2Ac in both control and LPS-conditioned cells resolved into four peaks (P1–P4), and as expected A subunit eluted in these same peaks (Fig. 1, E and F). Regulatory subunit B55 eluted in P1 and B56γ3 in P3–4, showing the separation of different heterotrimeric forms of PP2A. The major, distinctive difference between control and LPS-conditioned cells was a much larger peak of PP2Ac in P4 of conditioned cells (Fig. 1G). The amount of A subunit in P4 did not increase (Fig. 1H), consistent with the differences seen in analysis of whole cell extracts (Fig. 1B). These results revealed that endotoxin-conditioned cells contained a distinct biochemical species of PP2A that accounted for the significant increase in PP2Ac levels. Although the total cellular levels of PP2Ac increased only ~30%, there was a ~4-fold increase in the amount of P4.

PP2A Binds Directly to VCP/p97—To identify the novel PP2A complex in LPS-conditioned cells, fractions 40–45 from multiple preparations were pooled together and immunoprecipitated with anti-PP2Ac antibodies, and the recovered proteins were subjected to liquid chromatography/MS. We identified VCP/p97 from 127 total spectra and 23 unique peptides, providing 35% overall protein sequence coverage (Fig. 2A). We used reciprocal immunoprecipitations to demonstrate the association of endogenous PP2Ac and VCP/p97 proteins in unstimulated RAW264.7 cells (Fig. 2B) and primary human PBMCs (Fig. 2C). We found that LPS conditioning of PBMCs slightly increased the protein levels of both PP2Ac and VCP/p97 in whole cell extracts and increased co-precipitation of PP2Ac with VCP/p97 by 1.8-fold relative to controls (Fig. 2C). The PP2A A subunit was also recovered in the VCP/p97 immunoprecipitates from PBMCs, but after LPS conditioning of the cells, the recovery of A subunit did not increase proportionally with the recovery of PP2A (Fig. 2C). These data demonstrated association of endogenous PP2Ac with VCP/p97 in a murine cell line and in primary human cells and showed accumulation of the complex in cells in response to endotoxin conditioning.

To further characterize the PP2Ac complexes from LPS-conditioned RAW264.7 cells, we pooled and concentrated fractions 40–45 from DEAE chromatography, which contained PP2Ac, VCP/p97, A subunit, and B56γ3. Superoxide 12 gel permeation chromatography (Fig. 2D) partially resolved a major peak of PP2Ac that co-eluted with PP2A A subunit (centered at $V_e = 12$ ml, 100–200 kDa) from a minor peak that co-eluted with VCP/p97 (centered at $V_e = 10$ ml, ~500 kDa). We concluded that the first eluted peak was PP2A associated with the hexameric VCP/p97, whereas the second peak contained PP2Ac associated with its scaffold A subunit, as AC dimers and ABC trimers.

To demonstrate a direct protein-protein interaction, we used recombinant GST-VCP/p97 purified from bacteria in a GST pulldown assay with purified PP2A AC core dimer. Both the PP2Ac and A subunit bound to GST-VCP/p97, but not to GST used as a control (Fig. 2D), and the GST-VCP/p97-PP2A complex had PP2A phosphatase activity (supplemental Fig. S1). This showed that GST-VCP/p97 directly bound to the AC core dimer of PP2A without inhibiting the phosphatase. VCP/p97 Promotes iNOS-dependent Nitration of Tyr284 in PP2Ac—Recently, LPS plus IFN-γ stimulation of wild type but not iNOS knock-out mouse endothelial cells was reported to increase PP2A activity, which was correlated to the immunoblotting of 3-nitrotyrosine (3NT) in PP2Ac (17). We observed the formation of 3NT in immunoprecipitated PP2Ac following LPS plus IFN-γ stimulation of RAW264.7 cells (Fig. 3A). Mouse BMDMs were isolated and conditioned with LPS, which stimulated formation of 3NT in immunoprecipitated PP2Ac (Fig. 3B). This nitration of Tyr in PP2A was essentially absent in BMDMs from iNOS knock-out mice with or without LPS conditioning (Fig. 3B). Even though nitration of PP2Ac was essentially absent in iNOS knock-out BMDMs, co-precipitation of PP2Ac with VCP/p97 from wild type and iNOS knock-out BMDMs was increased to the same or a greater extent following LPS plus IFN-γ stimulation (Fig. 3C). This showed that nitration of PP2Ac was not required for association with VCP/p97 and did not interfere with the proteins forming a complex but probably occurred after formation of the complex with VCP/p97. Nitration of purified PP2A by reaction with peroxynitrite did not affect its binding to GST-VCP/p97 in a pulldown assay (supplemental Fig. S2).

Localized formation of highly reactive and short-lived peroxynitrite from NO plus superoxide is the most probable mechanism for formation of 3NT. We found iNOS co-precipitated
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To demonstrate that in live cells Tyr284 was the site of nitration in PP2Ac, we expressed Myc-PP2Ac wild type (WT) and Y284F in 293T cells. The cells were treated with peroxynitrite or an inactive, degraded peroxynitrite as control, and the extracts were prepared for immunoprecipitation of the Myc-PP2Ac. The Y284F protein had a drastically lower level of tyrosine nitration relative to WT, confirming that in cells Tyr284 was the primary site of nitration in PP2Ac (Fig. 3). Recovery of A subunit with Myc-PP2Ac WT was reduced ~30% by peroxynitrite treatment, compared with no decrease in A subunit association with Myc-PP2Ac Y284F (Fig. 3). We suspect that the reduced A subunit association with WT PP2Ac reflected the stoichiometry of Tyr284 nitration achieved in live cells. Taken together, these results support a model that VCP/p97 associates with core AC dimer of PP2A and recruits iNOS to promote nitration of Tyr284 in PP2Ac to cause release of the A subunit from the complex.

Overexpression of PP2Ac or VCP/p97 Is Sufficient to Reduce p38 MAPK Activation and TNF-α Production—We questioned whether transiently increasing the levels of PP2Ac or VCP/p97 was sufficient to mimic endotoxin conditioning. Low level transient overexpression of VCP/p97 or PP2Ac in RAW264.7 cells (supplemental Fig. S3) caused a significant reduction in the release of TNF-α in response to LPS stimulation (Fig. 4A). Because suppressed activation of MAP kinases is a hallmark of LPS conditioning, we assayed whether overexpression of VCP/p97 or PP2Ac altered phosphorylation of ERK, JNK, and p38 MAPK. Activation of p38 MAPK was both reduced in amplitude and delayed in onset (Fig. 4, B and C). JNK and ERK also were inhibited, with the relative effects p38 = JNK > ERK (supplemental Fig. S3B). Overexpression of PP2Ac and VCP/p97 together did not have an additive effect on p38 MAPK (data not shown). Thus, a transient increase in levels of either VCP/p97 or PP2Ac was sufficient to mimic the reduction in LPS-induced TNF-α release and MAPK activation that are characteristic of endotoxin-tolerant cells.

What might be the effect of Tyr nitration on PP2Ac? Peroxynitrite treatment of purified PP2A formed 3NT in PP2Ac (Fig. 3E) and caused dissociation of the AC heterodimer, based on the change in elution during Superose12 gel permeation chromatography (Fig. 3F). We performed MS/MS analysis of PP2A after reaction with peroxynitrite, and the collision-activated dissociation mass spectra of both the tryptic and Asp-N peptides mapped Tyr284 as the predominant nitration site (Fig. 3, G and H).

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**FIGURE 4.** PP2Ac-VCP/p97 inhibits p38 activation through DUSP1. A–C, RAW264.7 cells were transfected to express VCP/p97, HA−PP2Ac, or empty vector (Cont.) and cultured for 24 h. A, TNF−α released in the medium were measured by ELISA 4 h after treatment with LPS. The data are the means ± S.E. (n = 3). B, activation of p38 MAPK and MKK3/6 by LPS was analyzed by phospho-site immunoblotting at the indicated times. Representative images from three independent experiments are shown. C, quantitative analysis of p38 MAPK phosphorylation in B. D, RAW264.7 cells were treated with or without LPS (0.1 μg/ml) for 16 h, and the level of DUSP1 was quantified by immunoblotting. Representative images from two independent experiments are shown. E, cells were transfected to express VCP/p97, HA−PP2Ac, or empty vector and cultured for 24 h, and the level of DUSP1 was quantified by immunoblotting. Representative images from two independent experiments are shown. F, DUSP1 was immunoprecipitated from LPS-conditioned RAW264.7 cells and immunoblotted for PP2Ac, VCP/p97, and DUSP1. Normal IgG was used as control (Cont.). Representative images from two independent experiments are shown.

Conversely, pharmacological inhibition of PP2A or siRNA knockdown of VCP/p97 enhanced MAPK activation in response to LPS. Selective inhibition of type 2A phosphatase with okadaic acid enhanced the level and duration of p38 MAPK, JNK, and ERK phosphorylation in response to LPS stimulation of RAW264.7 cells (supplemental Fig. S3C). Knockdown of VCP/p97 by siRNA enhanced activation of p38 MAPK and JNK in response to LPS and also increased the level of polyubiquitinated proteins consistent with the known effects of VCP/p97 knockdown (supplemental Fig. S3, D and E).

**Elevation of DUSP1/MKP1 Levels by Endotoxin or PP2Ac-VCP/p97**—We focused on p38 MAPK to investigate how VCP/p97 and PP2Ac were able to mimic LPS conditioning. Activation of p38 MAPK depends on dual phosphorylation of the TGY motif, catalyzed by the MKK3/6 kinases (22). We detected phosphorylation of endogenous MKK3/6 with a site-specific antibody, but this did not change in response to overexpression of either PP2Ac or VCP/p97 (Fig. 4B). Alternatively, p38 MAPK activation could be restricted by an increase in MAPK phosphatases. Indeed, we found that LPS conditioning increased the level of dual specificity phosphatase (DUSP1/MKP1) in RAW264.7 cells (Fig. 4D). Transient overexpression of either PP2Ac or VCP/p97 also increased the protein levels of DUSP1 (Fig. 4E). The mRNA levels of DUSP1 in these cells were the same by reverse transcription-PCR (supplemental Fig. S3F), consistent with post-transcriptional mechanisms of regulation. Moreover, endogenous DUSP1 was recovered with PP2Ac and VCP/p97 by immunoprecipitation (Fig. 4F). These results show that LPS conditioning stably increased DUSP1 protein levels, and transient overexpression of either VCP/p97 or PP2Ac was sufficient to mimic this response.

**DISCUSSION**

Endotoxin tolerance is an adaptive response of cells that renders them refractory to stimulation through TLRs. This study provides new evidence that connects phosphatase PP2A to the actions of VCP/p97, an abundant chaperone protein that is also linked to regulation of ubiquitination and protein degradation via the 26 S proteasome. We show that endogenous VCP/p97 forms a complex with PP2Ac and also associates with iNOS, which was required for Tyr nitration of PP2Ac. Nitration was mapped to Tyr284 in PP2Ac and shown to release the A subunit, which otherwise represses phosphatase activity. We propose (Fig. 5) that in cells a fraction of PP2A associates with VCP/p97, and in the hours following TLR activation there is synthesis of iNOS that is recruited to nitrate PP2Ac at Tyr284. The 3NT-PP2Ac remains sequestered by iNOS to induce Tyr284 nitration of PP2Ac that leads to AC dimer dissociation to result in PP2Ac-VCP/p97 complex formation with high phosphatase activity. One of the targets of the PP2A-VCP/p97 complexes is DUSP1. Increased PP2A-VCP/p97 complexes augment DUSP1 levels that decrease p38 MAPK activation and cytokine production in response to secondary stimuli.
amount of PP2Ac in conditioned cells was increased by only ~30%, but this was notable because the PP2Ac levels are under such stringent auto-regulatory control. The surplus PP2Ac was purified as a novel high Mₙ complex with VCP, also known simply as p97. VCP/p97 is a member of the AAA class of ATPase and functions as a chaperone to process polyubiquitinated proteins. VCP/p97 determines whether polyubiquitinated proteins undergo proteasome degradation or are deubiquitinated enzymatically and spared (18). Unstimulated cell lines and primary cells have a pre-existing pool of PP2A-VP/p97 complex. Endotoxin conditioning of primary cells increased the amount of PP2Ac-VP/p97 complex recovered by immunoprecipitation. We propose that accumulation of this complex has an important role in endotoxin tolerance, because overexpression of either protein was sufficient to partially mimic tolerance in terms of a suppressed activation of p38 MAPK and reduced release of TNF-α.

PP2A-VP/p97 Complex Inhibits p38 MAPK Activation by Increasing Levels of DUSP1—Suppression of p38 MAPK activation did not involve reduction in phosphorylation of MKK3/6, suggesting that regulation was occurring on the phosphatase side of the equation. DUSP1/MKP1 is critical for attenuation of p38 MAPK and JNK signaling during innate immune responses to LPS, and indeed the DUSP1 KO mouse shows increased sensitivity to endotoxin shock induced by LPS (23, 24). Increased DUSP1 levels have been seen in the LPS-conditioned THP-1 human monocyte-like cell line (25). The LPS-induced increase in DUSP1 has been shown to involve both transcriptional and post-translational mechanisms (23). DUSP1 levels also increase in response to various stress stimuli, providing a negative feedback reaction that limits inflammation. Dexamethasone induces DUSP1 transcription and protein stabilization, leading to reduced cytokine release, accounting in part for the anti-inflammatory effects of the steroid (26, 27). Transient overexpression of PP2Ac or VCP/p97 caused accumulation of DUSP1 protein, without a change in mRNA, suggesting that this response involved modification of the protein.

Contradictory reports claim that Ser/Thr phosphorylation of DUSP1 by ERK causes either an increase (28, 29) or a decrease (30) in the degradation of DUSP1. We could co-immunoprecipitate DUSP1 with both PP2Ac, the Ser/Thr phosphatase, and with VCP/p97, a chaperone that controls access to the proteasome. Our view is that rescue of DUSP1 from proteasomal degradation could arise from a combination of direct dephosphorylation by PP2Ac and deubiquitination by a VCP/p97-associated USP enzyme (18). Experiments to test these possibilities are difficult because of the low levels and rapid degradation of DUSP1. Another possible mechanism to regulate DUSP1 polyubiquitination and proteasome degradation is negative regulation of SCF-Skp2 E3 ligase (28, 29). Skp2 phosphorylation by Akt triggers SCF complex formation and E3 ligase activity (31). It is possible that PP2A-VP/p97 inhibits DUSP1 polyubiquitination by dephosphorylating Skp2. Our results assign a new function to VCP/p97 as a regulatory protein that activates one phosphatase (PP2A) to increase the levels of another phosphatase (DUSP1/MKP1) and position VCP/p97 as a key factor in tolerance.

Nitration of Tyr₂⁸⁴ in PP2Ac and Dissociation of AC Dimer—Even though we found that VCP/p97 binds PP2A AC dimer in cells and in biochemical assays with purified proteins, we isolated from preconditioned cells a PP2Ac-VP/p97 complex that lacked detectable PP2A A subunit. Post-translational modifications are known to regulate PP2A holoenzyme composition and activity, so we speculated that some modification of PP2Ac or PP2A A subunit could cause AC dimer dissociation. We found increased levels of 3NT in PP2Ac from endotoxin-conditioned versus control RAW264.7 cells. We did not detect any 3NT in PP2A A subunit immunoprecipitated from endotoxin-conditioned cells (data not shown). VCP/p97 co-precipitated iNOS and PP2Ac, suggesting that it acted as a scaffold to assemble a three-way complex for nitration of PP2Ac. In conditioned BMDMs from iNOS knock-out mice, there was still a trace of 3NT in PP2Ac, indicating that the primary source of peroxynitrite was iNOS, but there also may be an iNOS-independent pathway.

We used purified AC dimer reaction with peroxynitrite in vitro to map by MS/MS the nitration site in PP2Ac primarily at Tyr₂⁸⁴, and coincidently discovered that this reaction dissociated the A subunit from PP2Ac. Because association with A subunit allosterically decreases the reaction velocity of PP2Ac (32), dissociation of AC dimers will increase PP2A activity. Tyr₂⁸⁴ in PP2Ac is located near the interface with the A subunit (supplemental Fig. S4A) and is conserved among human, mouse, chicken, frog, zebra fish, and nematode, but not Drosophila or yeast (supplemental Fig. S4B). There is not a Tyr residue at the corresponding position in either PP4 or PP6, predicting that the reaction will not prove to be common to other type 2A phosphatases (supplemental Fig. S4C). PP2Ac is reported to be phosphorylated on Tyr₂⁸⁴ in v-Src transformed cells (16), confirming that this residue is surface-exposed in PP2A holoenzymes in live cells and raising the possibility of alternative nitration or phosphorylation, depending on the stimuli. Nitration of Tyr is not readily reversible and we speculate that the PP2Ac-Tyr₂⁸⁴-3NT is stably sequestered with VCP/p97 because it is unable to bind the A subunit scaffold. This would account for accumulation of PP2Ac-VP/p97 complex in endotoxin-conditioned cells and elevation of the PP2Ac levels.

Reciprocal Regulation between PP2Ac and VCP/p97—PP2Ac mediates dephosphorylation and activation of VCP/p97 as an ATPase. VCP/p97 ATPase is negatively regulated by Akt phosphorylation (33, 34). We phosphorylated recombinant VCP/p97 by purified Akt and assayed PP2A reactivation of the ATPase (supplemental Fig. S5A). This activation involved dephosphorylation of sites in VCP/p97 that were detected by phospho-Akt substrate immunoblotting (supplemental Fig. S5B). Akt phosphorylation of VCP/p97 is known to reduce association with ubiquitinated proteins (33). Therefore this PP2A dephosphorylation would activate VCP/p97 association with ubiquitinated proteins, in parallel with activation of the ATPase activity. Thus, we propose that formation of PP2Ac-VP/p97 complexes involved mutual activation of these component enzymes.

The effect of ATPase activation of VCP/p97 on proteasomal degradation is controversial. It has been proposed that VCP/
p97 targets multi-ubiquitin chains for elongation and is thereby required for ubiquitin-proteasome degradation (35, 36). Consistent with this, knockdown by siRNA or expression of an ATPase-deficient mutant of VCP/p97 causes significant accumulation of ubiquitinated proteins without affecting proteasome activity (37, 38). However, more recent observations revealed that VCP/p97 also associates with deubiquitinating enzymes to promote substrate deubiquitination (39, 40), which leads to diverting proteins from proteasomal degradation. These studies suggest a paradigm in which VCP/p97 plays a role in the fate of its client proteins (18). Activation of VCP/p97 by PP2A dephosphorylation may increase or decrease protein turnover, with the direction being substrate-dependent. Discovery of PP2A association with VCP/p97 connects the proteosome.

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