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Protein Phosphatase 2A and Neutral Sphingomyelinase 2 Regulate IRAK-1 Protein Ubiquitination and Degradation in Response to Interleukin-1β*[S]

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The IL-1β signaling cascade is initiated by the phosphorylation of IL-1β receptor-associated kinase-1 (IRAK-1), followed by its ubiquitination and degradation. This paper investigates the regulation of IRAK-1 degradation in primary hepatocytes and in HEK cells overexpressing the IL-1β receptor. We provide evidence that protein phosphatase 2A (PP2A) is a negative regulator of the phosphorylation, Lys^{48}-linked ubiquitination, and degradation of IRAK-1. PP2A catalytic activity increased within 30 min of stimulation with IL-1β. siRNA against PP2A catalytic subunit (PP2Ac) or treatment with pharmacological inhibitor, okadaic acid, enhanced IRAK-1 Lys^{48}-linked ubiquitination and degradation. Direct interaction between PP2Ac and IRAK-1 was observed, suggesting that IRAK-1 might be a PP2A substrate. The mechanisms of PP2A activation by IL-1β involved neutral sphingomyelinase-2 (NSMase-2) and an accumulation of ceramide. Overexpression of NSMase-2 delayed IRAK-1 degradation in a PP2A-dependent manner, whereas NSMase-2 silencing had the opposite effect. The addition of sphingomyelinase, ceramide, or a proteasome inhibitor all led to retention of IRAK-1 at the cell membrane and to increased JNK phosphorylation. This study suggests that NSMase-2- and PP2A-dependent regulation of IRAK-1 degradation is a novel mechanism to fine tune the magnitude of IL-1β response.

The IL-1β receptor type I (IL-1RI) belongs to the Toll-like/IL-1 receptor (TIR)[2] superfamily and mediates cellular responses to IL-1β (1, 2). A ligand-induced dimerization of IL-1RI and IL-1β receptor accessory protein leads to the formation of active receptor complex, including the adapter protein myeloid differentiation factor 88 (MyD88), the interleukin-1 receptor-associated kinase (IRAK)-1, IRAK-4, and the ubiquitin ligase TRAF-6 (3–5). Phosphorylation of IRAK-1 is essential for downstream signaling. Initially, IRAK-1 is phosphorylated at Thr^{209}, Thr^{387}, and Ser^{376} by IRAK-4 (6–8). This causes stimulation of the IRAK-1 activity and a cascade of autophosphorylation events at residues within the N-terminal proline-, serine-, and threonine-rich (ProST) region of IRAK-1 (9, 10). The resulting hyperphosphorylation leads to the dissociation of IRAK-1 and TRAF-6 from the rest of the receptor complex and the formation of an intermediary complex at the plasma membrane, which also contains the transforming growth factor-β-activated kinase-1 (TAK-1) (6, 7, 11, 12). The latter is essential for the activation of downstream transcription factors, such as nuclear factor-κB (NF-κB) through the IκB kinases and activator protein-1 through JNK and p38 (13).

It is well established that the IL-1β pathway diverges at the level of IRAK-1. During signaling, IRAK-1 undergoes complex modifications, including phosphorylation and ubiquitination at multiple residues. Some but not all of the phosphorylated residues have been identified: Thr^{66} (10), Thr^{100} (14), Ser^{131} (9), Thr^{209} (6), Ser^{371} (9), Ser^{376} (8), Thr^{387} (6, 8), and Ser^{568} (15). The roles of these sites in the signaling pathway are diverse, and many seem to target ubiquitin ligases. Recently, Lys^{134}, Lys^{180} (16, 17), Lys^{355}, and Lys^{397} (18) within the ProST region and the kinase domain of IRAK-1 have been shown to undergo polyubiquitination. Lys^{180} is predominantly used for Lys^{63}-linked polyubiquitination (17), whereas Lys^{134} is a competing site for both Lys^{63}- and Lys^{48}-linked polyubiquitination (16). The Lys^{63}-dependent polyubiquitination of IRAK-1, which is catalyzed by TRAF-6 and the Pellino ubiquitin ligases (17, 19, 20), mediates the formation of protein-protein complexes that are required for NF-κB activation but are expandable for JNK activation (21–23). In contrast, Lys^{48}-dependent polyubiquitination targets IRAK-1 to the proteasomes for degradation (16).

The degradation of IRAK-1 is shown to down-regulate TIR-mediated signaling by limiting the availability of IRAK-1 (24) and to determine the magnitude of the response to several ligands of the TIR family, including the bacterial endotoxin LPS. A reduction in IRAK-1 protein in LPS-tolerant cells, for example, is a major mechanism for the onset of endotoxin tolerance, the phenomenon of decreased inflammation in cells or organisms after pre-exposure to LPS (25). *Vice versa*, stabilization of IRAK-1 molecule by pharmacological agents up-regulates LPS response in macrophages (26), whereas IFN and GM-CSF prevent endotoxin tolerance in monocytes by increasing IRAK-1 and its association with MyD88 (27). Hence, IRAK-1

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[2] The abbreviations used are: TIR, Toll-like/IL-1 receptor; SMase, sphingomyelinase; bSMase, bacterial sphingomyelinase; C_6-NBD-SM, 6-*N*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino-sphingomyelin; Lys^{48}-Ub, antibody specific for Lys^{48}-dependent polyubiquitin chains; MyBP, myelin basic protein; NF-κB, nuclear factor κB; NSMase-2, neutral sphingomyelinase-2; OkAc, okadaic acid; TRITC, tetramethylrhodamine isothiocyanate; ProST, proline-, serine-, and threonine-rich; IP, immunoprecipitation.

proteasomal degradation is critical for the magnitude of cellular response, yet the factors controlling the rate of IRAK-1 phosphorylation, Lys^{48}-dependent ubiquitination, and degradation are not well understood.

Previously, it was shown that IL-1β transiently stimulates the hydrolysis of plasma membrane sphingomyelin in various cell types (28–31). Neutral sphingomyelinase-2 (NSMase-2) was identified as the *bona fide* IL-1β-inducible sphingomyelinase, which is localized at the plasma membrane (32). The precise role of NSMase-2 in the cascade, however, turned out to be a complex one. Our earlier studies found that overexpression of NSMase-2 in primary rat hepatocytes kept IRAK-1 in less phosphorylated and less ubiquitinated form, leading to more potent and prolonged JNK activation. *Vice versa*, silencing or inhibiting of NSMase-2 led to a more rapid degradation of IRAK-1 and inhibited IL-1β-induced JNK activation by 70–80% (32, 33). These results indicated that NSMase-2 activation might serve to amplify the IL-1β response by negatively regulating the rate of IRAK-1 degradation during signaling.

NSMase-2 is involved in various cellular responses to inflammation (34–38). Abnormal basal activity of NSMase-2 is seen in pathophysiological conditions like aging and cancer, as well as during oxidative stress (33, 39–41), whereas the product of NSMase-2, ceramide, has been implicated in the regulation of JNK, NF-κB, PKCζ, Akt-1, and other signaling molecules (42–46). The protein phosphatases of the PP2A and PP1 families are among the best characterized direct targets of ceramide (47, 48). Ceramide has been shown to bind the catalytic and the regulatory subunit of PP2A and to stimulate the catalytic activity of the phosphatase by inducing conformational changes. Activation of PP2A and other ceramide-activated protein phosphatases by ceramide has been implicated in the regulation of c-Myc, apoptosis, Rb, and SR proteins (49–54).

Protein phosphatases participate in the IL-1β signaling pathway mainly as negative regulators. PP2A, in particular, is shown to dephosphorylate RelA (55), IκB kinase (56), and certain MAPKs (57), thus suppressing the magnitude of the response. Two other phosphatases, PP2C and PP6, similarly suppress JNK and NF-κB activation by binding and dephosphorylating TAK-1 (58, 59). In this paper, we report that PP2A also acts as a positive regulator in the IL-1β cascade that binds and dephosphorylates IRAK-1, thus inhibiting Lys^{48}-linked IRAK-1 ubiquitination and degradation. Our results infer the existence of a positive feedback mode of regulation in the IL-1β cascade.

EXPERIMENTAL PROCEDURES

Materials—Male Fisher 344 rats (150–200 g) were purchased from Harlan, Inc. (Indianapolis, IN). Animals received humane care according to the criteria outlined in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. HEK 293 cells overexpressing the IL-1β receptor Type I (293-IL-1RI) were a gift from Dr. X. Li (Cleveland Clinic, Cleveland, OH). Rat and human recombinant IL-1β were from Invitrogen. Tet system-approved FBS was from BD Biosciences Clontech (Palo Alto, CA), and growth factor-reduced Matrigel® was from BD Biosciences Discovery Labware (Bedford, MA). 6-*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)amino-sphingomyelin (C_6-NBD-SM) was from Molecular Probes, Inc. (Eugene, OR). MG-132, MG-115, and okadaic acid (OkAc) were from BIOMOL Research Laboratory (Plymouth Meeting, PA). Anti-phospho-JNK1/2 and anti-ubiquitin (P4D1) antibodies were from Cell Signaling (Beverly, MA). Anti-FLAG, anti-β-actin, and all secondary antibodies were from Sigma. Anti-IRAK-1 (polyclonal rabbit and monoclonal mouse) antibodies and protein A- and G-agarose beads were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The antibody against Lys^{48}-linked ubiquitin chains (clone Apu2) was from Millipore (Billerica, MA), whereas anti-PP2A catalytic subunit (PP2Ac) antibody was from Upstate Inc. (Lake Placid, NY). Rhodamine-conjugated wheat germ agglutinin was from Vector Laboratories Inc. (Burlingame, CA), and the FITC-conjugated goat anti-rabbit IgG antibodies were from Jackson Immunoresearch Laboratories (West Grove, PA). Myelin basic protein (MyBP) and recombinant PKA were from Sigma, whereas [γ-^{32}P]ATP (10 Ci/mmol, 2 mCi/ml) was from PerkinElmer Life Sciences. λ-Protein phosphatase was from New England Biolabs (Beverly, MA), bacterial SMase (bSMase; *Staphylococcus aureus*) was from Sigma, and *N*-acetylsphingosine was from Avanti Polar Lipids (Alabaster, AL). Murine NSMase-2-specific siRNA (AATGCTACTGGCTGGTGGACC), human PP2Ac-specific siRNA (CTCGTGGAAACCACACTCAAA), and scrambled control siRNA (catalog no. 1027283) were from Qiagen (Valencia, CA).

Adenoviral Constructs—Adenovirus expressing FLAG-tagged mouse NSMase-2 (Ad-NSMase-2) was purified as described previously (32). DNA oligonucleotides encoding a sense-loop-antisense sequence (GCCCTCATCTTCCCATGTTACTTCAAGAGAGTAACATGGGAAGATGAGGGC) against the rat NSMase-2 and a scrambled sense-loop-antisense RNA sequence (scr) were subcloned into pENTR/U6 entry vector (Invitrogen), creating an RNAi cassette, which was subcloned into pAdTrack vector that also encodes GFP (gift from Dr. George Smith, University of Kentucky). Homologous recombination between the pAdTrack and the adenoviral backbone plasmid (pAdEasy-1) was done in *Escherichia coli* strain BJ5183 to produce adenovirus expressing the shRNA against NSMase-2 (Ad-sh-NSMase-2) or the corresponding scrambled sequence (Ad-scr).

Cell Cultures and Treatments—Hepatocytes were isolated from ether-anesthetized male Fisher 344 rats and cultured in Matrigel-coated dishes as described previously (33). 293-IL-1RI cells were maintained in DMEM supplemented with 10% FBS. Infections with Ad-NSMase-2, Ad-sh-NSMase-2, or Ad-scr were performed 48 h after hepatocyte isolation, at a multiplicity of infection between 2 and 5. When necessary, the expression of the transgene was induced by the addition of doxycycline at the day of infection and again 48 h later. Transfections with siRNA (100 nM) were done on day 3 of hepatocyte culture using Lipofectamine Plus reagent. 293-IL-1RI cells (75–90% confluent) were infected or transfected according to the same protocols, with the exception that siRNA was added at final concentration of 50 nM.

Cells were treated with IL-1β 72 h after infection. Inhibitors (or appropriate vehicles) were added 30 min before the treatment with IL-1β at the indicated concentrations. OkAc was used at a final concentration of 10 nM, at which it is highly selective for PP2A (IC_{50} = 0.51 nM) but has no effect on PP1

(IC_{50} = 42 nM), PP2B (IC_{50} = 5000 nM), or PP2C (IC_{50} ≫10,000 nM).

Preparation of Cell Extracts—To harvest cultured primary hepatocytes, the medium was first aspirated, and the Matrigel was reliquified by incubating with PBS containing 5 mM EDTA for 30 min at 4 °C. 293-IL-1RI cells were harvested in cold PBS using cell scrapers. The collected cells were pelleted by centrifugation at 500 × *g* for 4 min, rinsed, and incubated with 50–200 μl of lysis buffer (1 mM EDTA, 0.5% Triton X-100, 1 mM Na_2VO_4, 1 mM NaF, 1:200 (v/v) protease inhibitor mixture, 10 mM Tris-HCl, pH 7.4) on ice for 30 min. Cell lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C. The clear supernatant was used for SDS-PAGE and Western blot analyses.

SDS-PAGE, Western Blotting, and Immunoprecipitation— Proteins were resolved by 10% SDS-PAGE and transferred to Immobilon-P polyvinylidene fluoride membrane by semidry blotting. Specified proteins were detected using the antibodies described under "Materials." Protein-antibody interactions were visualized using the ECF kit (Amersham Biosciences) and a Storm860 fluorescent scanning instrument, and analyzed using ImageQuant5.0 software (GE Healthcare). In the immunoprecipitation experiments, 293-IL-1RI cells (10^7 cells/sample) were lysed in a buffer containing 150 mM NaCl, 1.5 mM $MgCl_2$, 2 mM EDTA, 2 mM DTT, 0.5% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, and protease inhibitor mixture in 20 mM Hepes, pH 7.4. The extracts were incubated overnight with anti-IRAK-1, anti-PP2Ac, or anti-Lys^{48}-linked ubiquitin antibodies and prewashed protein A-agarose or G-agarose slurry. After collecting the sample by centrifugation, the immunocomplexes bound to protein A- or G-agarose were washed three times in PBS and subjected to SDS-PAGE. The immunoprecipitated IRAK-1 or PP2A was visualized by Western blot using specific polyclonal anti-IRAK-1 or monoclonal anti-PP2Ac antibodies. Co-immunoprecipitation was detected after washing the membranes with Tween 20 (0.5%) in PBS and reprobing with anti-PP2A or anti-IRAK-1, respectively.

NSMase Activity Assay—Cells from each dish were harvested, resuspended in lysis buffer (see above) without Triton X-100, lysed with three consecutive freeze-thaw cycles, and homogenized by sonication for 5 min. NSMase activity was determined as described previously, using C_6-NBD-SM as a substrate (28, 60).

In Vitro Serine/Threonine Phosphatase Assay—The activity of serine/threonine phosphatases was quantified using ^{32}P-labeled myelin basic protein (^{32}P-MyBP) as a substrate. MyBP was phosphorylated for 18 h at 30 °C with 50 μCi of [^{32}P]ATP in the presence of PKA catalytic subunit (50 units). The reaction was terminated by the addition of TCA, and ^{32}P-MyBP was purified by dialysis. MyBP was phosphorylated on serine/threonine residues to high stoichiometries (routinely 2 nmol of P_i/1 nmol of MyBP). The phosphatase reaction was carried out for 10 min at 30 °C in 50 mM Tris-HCl (pH 7.0) containing 15 μM ^{32}P-MyBP, 10 μg of cell protein, 0.1 mM EDTA, 5 mM DTT, and 0.01% Brij35 (final volume of 50 μl). The reaction was stopped by the addition of TCA, and the radioactivity released in the supernatant was counted on a scintillation counter. The specific PP2A activity was calculated as the difference between the total phosphatase activity and that measured in the presence of 10 nM OkAc (61).

Indirect Immunofluorescence—293-IL-1RI cells were cultured on coverslips to 90% confluence and treated as indicated. Cells were fixed with 3% paraformaldehyde and permeabilized with 0.2% Triton X-100. TRITC-conjugated wheat germ agglutinin was used as a marker for plasma membrane and was applied for 20 min before the permeabilization of the cells. The endogenous IRAK-1 was labeled with polyclonal anti-IRAK-1 antibody. The immune complexes were visualized with FITC-conjugated goat anti-rabbit IgG. Cells were mounted on slides in glycerol buffer containing Hoechst 33342 for nuclear staining. Incubations with secondary antibodies alone were used as control for nonspecific fluorescence. Slides were examined on a Leica LCS confocal laser-scanning microscope.

Statistical Analysis—After proving the assumption of equal variance across groups, statistical significance was determined by Student's *t* test. Each experiment was reproduced at least three times. The data are reported as mean ± S.D. A *p* value of less then 0.05 was considered significant.

RESULTS

Patterns of Activation of IRAK-1 and JNK in 293-IL-1R Cells and in Primary Rat Hepatocytes—IL-1β signaling cascade was studied in 293-IL-1RI cells, a commonly used transgenic model and in primary rat hepatocytes, a physiologically relevant system. As shown in Fig. 1, the response elicited by IL-1β in the transgenic cells (Fig. 1*B*) was similar to that observed in the primary cells (Fig. 1*A*). The IL-1β treatment led to activation of JNK, as judged by the appearance of phosphorylated JNK forms on a Western blot (Fig. 1, *A* and *B*). IRAK-1, in turn, appeared as a single band with a molecular mass of 80 kDa in non-stimulated cells. The addition of IL-1β led to the appearance of a diffused pattern of multiple IRAK-1 forms (hyperphosphorylated and mono- or polyubiquitinated (18, 24)) with higher molecular mass. It was paralleled by the disappearance of the 80 kDa band (Fig. 1, *A* and *B*).

The observed activation of IRAK-1 was swift, with less then 10% of non-modified IRAK-1 molecules visible 30 min after IL-1β stimulation (Fig. 1, *A–D*). This loss was blunted in part by MG-132, a proteasome inhibitor (Fig. 1, *E* and *F*), indicative of ongoing degradation of IRAK-1 in the proteasomes (19, 24). As anticipated, the suppression of proteasomal functions led to an accumulation of modified IRAK-1 forms. A parallel accumulation of non-modified IRAK-1 was also observed, which is consistent with the reversible nature of mono- and diubiquitination.

IRAK-1 Is Dephosphorylated during IL-1β Signaling in a PP2A-dependent Manner—PP2A activity increased transiently following treatment with IL-1β. Maximum effect was observed 30 min after the addition of the cytokine (Fig. 2). To test the role of PP2A in the IL-1β signaling cascade, cells were pretreated with 10 nM OkAc. At this concentration, OkAc specifically inhibits phosphatases from the PP2A family and has little effect on PP1, PP2B, or PP2C (62, 63). Alternatively, the expression of PP2A catalytic subunit was blocked using siRNA. OkAc treatment facilitated the disappearance of IRAK-1 and reversed the effects of MG-132 (Fig. 3, *A* and *B*). This might indicate that

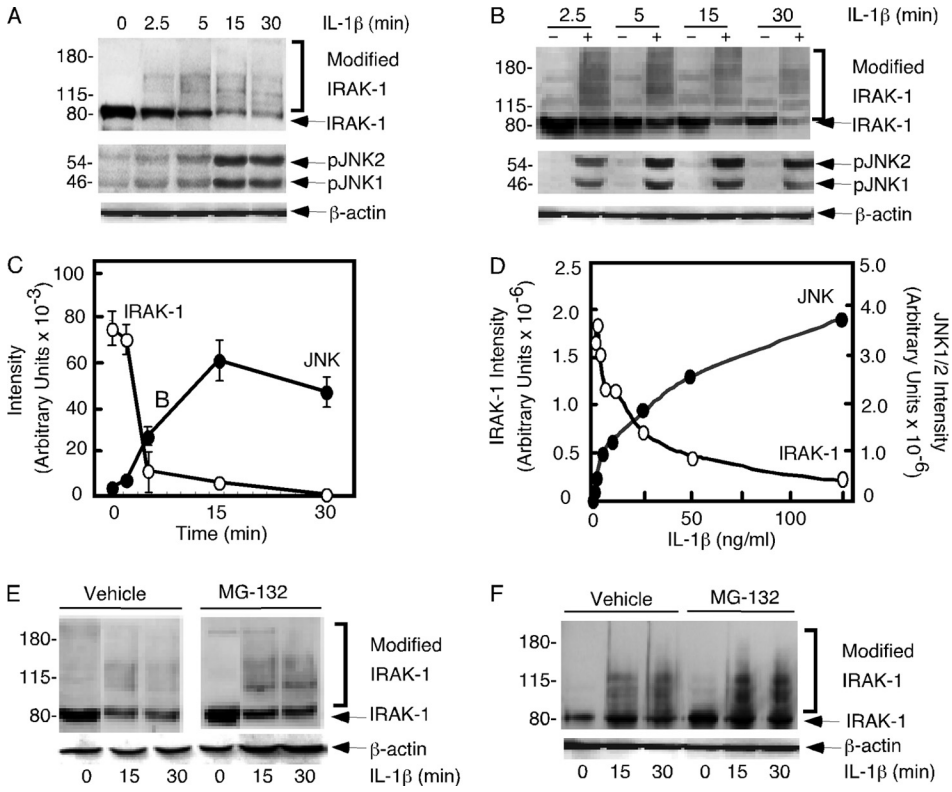

FIGURE 1. **IL-1β induces IRAK-1 degradation and JNK activation in primary rat hepatocytes and 293-IL-1RI cells.** Primary rat hepatocytes were treated with 25 ng/ml rat IL-1β for the indicated times (*A*, *C*, and *E*) or with the indicated doses for 30 min (*D*). 293-IL-1RI cells were stimulated with 25 ng/ml human IL-1β for the indicated times (*B* and *F*). MG-132 was added 30 min before the treatment with IL-1β at a concentration of 50 μM (*E* and *F*). IRAK-1 modification and JNK phosphorylation were determined by Western blotting using an antibody against IRAK-1 or the dually phosphorylated, active forms of JNK1 and JNK2. Quantification of the intensity of phospho-JNK1 and -JNK2 bands and that of the IRAK-1 80 kDa band was done on Storm860 scanning instrument. β-Actin was used as a control for uniform loading. Values are means ± S.D. (*error bars*), $n = 3$. Results are representative of more than five experiments.

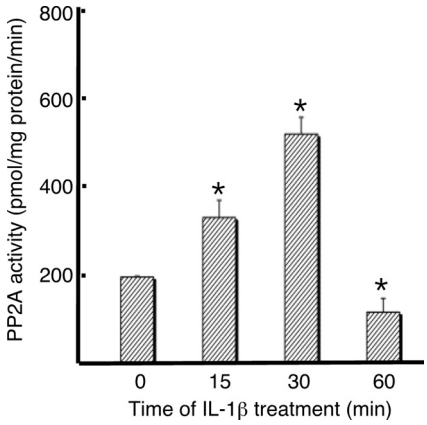

FIGURE 2. **IL-1β stimulates the catalytic activity of PP2A.** 293-IL-1RI cells were stimulated with human IL-1β (25 ng/ml) for the indicated times. Phosphatase activity was measured using ^{32}P-MyBP as an exogenous substrate in the presence and absence of 10 nM OkAc. OkAc-sensitive activity, which accounted for 30–40% of the total activity, was attributed to PP2A. Values are expressed as pmol/mg cell protein/min and are means ± S.D. (*error bars*), $n = 3$. Results are representative of four different cultures. *, $p \leq 0.05$.

IRAK-1 undergoes active dephosphorylation that delays its ubiquitination and degradation. However, the anticipated accumulation of highly modified IRAK-1 in the presence of MG-132 and OkAc was difficult to observe consistently. This, however, could be explained by a decline in the affinity of the IRAK-1 antibody with increasing ubiquitination of IRAK-1. To address this, we performed characterization of the antibody (supplemental Fig. 1) using as a reference an antibody specific for Lys^{48}-dependent polyubiquitin chains (Lys^{48}-Ub). The results of these experiments showed that the IRAK-1 antibody could be used efficiently for a Western blot analysis of modified IRAK-1 forms only after immunoprecipitation of these forms with Lys^{48}-Ub (supplemental Fig. 1*B*). These studies also confirmed that the affinity of the antibody declines with advanced IRAK-1 modification, and anti-IRAK-1 antibody was only efficient in immunoprecipitating the non-modified and not the modified IRAK-1.

To test directly whether PP2A regulates the modification of IRAK-1, the latter was immunoprecipitated with Lys^{48}-Ub. Western blot analyses of these immunoprecipitates with anti-IRAK-1 antibody revealed that OkAc as well as siRNA against PP2A substantially increased the abundance of Lys^{48}-linked ubiquitinated IRAK-1 (Fig. 3, *C* and *D*). These effects were especially pronounced in cells where PP2A was inhibited using siRNA, despite the fact that the efficiency of silencing was typically around 50%. Together, these data show that PP2A suppresses Lys^{48}-dependent IRAK-1 ubiquitination and suggest that it might dephosphorylate IRAK-1 at residues that are important for the targeting of the Lys^{48}-dependent ubiquitin ligases.

Co-immunoprecipitation of IRAK-1 and the PP2Ac—To investigate whether IRAK-1 and PP2Ac interact, IRAK-1- or PP2Ac- mediated immunoprecipitation (IP) was performed in lysates from 293-IL-RI cells. The immunoprecipitates were

analyzed by Western blotting with anti-IRAK-1 (Fig. 4*A*, *top*) or anti-PP2A (Fig. 4*B*, *top*) antibodies, followed by washing and reprobing with anti-PP2A (Fig. 4*A*, *bottom*) and anti-IRAK-1 (Fig. 4*B*, *bottom*) antibodies. When the endogenous IRAK-1 was immunoprecipitated (Fig. 4*A*), PP2Ac was efficiently pulled down from both IL-1β-treated and non-treated cells (Fig. 4*A*, *bottom*). Conversely, IRAK-1 co-immunoprecipitated with PP2Ac when anti-PP2Ac antibody was used for IP (Fig. 4*B*). Control IP reactions using mouse IgG or agarose beads confirmed the specificity of the observed interactions (Fig. 4*C*). It is of note that on a Western blot, the amount of IRAK co-immunoprecipitated with PP2Ac seemed significantly smaller (ratio of IRAK-1 to PP2Ac of 1:10) than the amount of PP2Ac co-immunoprecipitated with IRAK-1 (ratio appears to be 1:1). This signifies that only a small fraction of the cellular PP2Ac interacts with the endogenous IRAK-1 pool and is consistent with the existence of many other PP2A-interacting partners. Furthermore, the IRAK-1/PP2Ac interaction was constitutive, indicating that IL-1β stimulation affected PP2Ac catalytic activity but not IRAK-1 binding. This is in agreement with the observed increase in the *in vitro* measured specific activity of PP2A (Fig. 2).

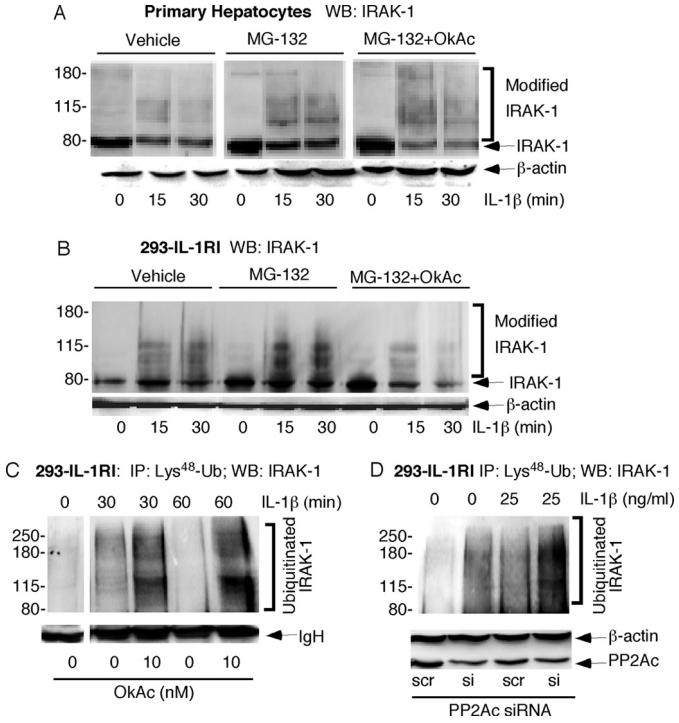

FIGURE 3. **PP2A affects the modification and Lys^{48}-dependent ubiquitination of IRAK-1.** Primary rat hepatocytes (*A*) or 293-IL-1RI cells (*B–D*) were treated with rat or human IL-1β (25 ng/ml) for the indicated times in the presence or absence of an inhibitor of proteasomes, MG-132 (50 μM), an inhibitor of PP2A, OkAc (10 nM), siRNA (*si*) against PP2A, or scrambled (*scr*) controls as indicated. *A* and *B*, Western blot (*WB*) analyses of IRAK-1 in total cell lysates using antibodies against IRAK-1. *C* and *D*, Western blot analyses of IRAK-1 using antibodies against IRAK-1 after IP with antibody against Lys^{48}-linked polyubiquitin chains. The efficiency of PP2A silencing was quantified by Western blotting using antibody against the catalytic subunit of PP2A. β-Actin was used as a control for uniform loading. Data are representative of four independent experiments.

PP2A Acts Downstream of NSMase-2—IL-1β also stimulates the activity of NSMase, causing an accumulation of ceramide. The latter is known to bind and activate PP2A (28, 47, 61, 64). Indeed, IL-1β treatment led to potent stimulation of NSMase activity in primary hepatocytes (Fig. 5*A*) and in 293-IL-1RI cells (data not shown), following a time course that was similar to that of PP2A activation. The increase in activity was attributed to NSMase-2 because it was completely inhibited by shRNA against this SMase form.

The level of NSMase-2 activity in the cells had a potent effect on IRAK-1 modifications. Overexpression of NSMase-2 attenuated the degradation of IRAK-1 induced by IL-1β stimulation (Fig. 5, *B* and *C*). *Vice versa*, NSMase-2 silencing led to the opposite effect (Fig. 5, *D* and *E*). These results suggested that NSMase-2 and PP2A played similar roles in the IL-1β cascade. Overexposed IRAK-1 immunoblots (Fig. 5*F*) revealed that the abundance of a band with an apparent molecular mass of 115 kDa was significantly suppressed in cells overexpressing NSMase-2 (and having high PP2A activity, as shown below). Notably, the abundance of seemingly the same band was elevated in cells with silenced PP2A (Fig. 5*G*); these differences disappeared when the extracts were treated *in vitro* with bacteriophage phosphatase (supplemental Fig. 2). The nature of the 115 kDa band is not completely clear at present; however, it might represent an IRAK-1 form whose

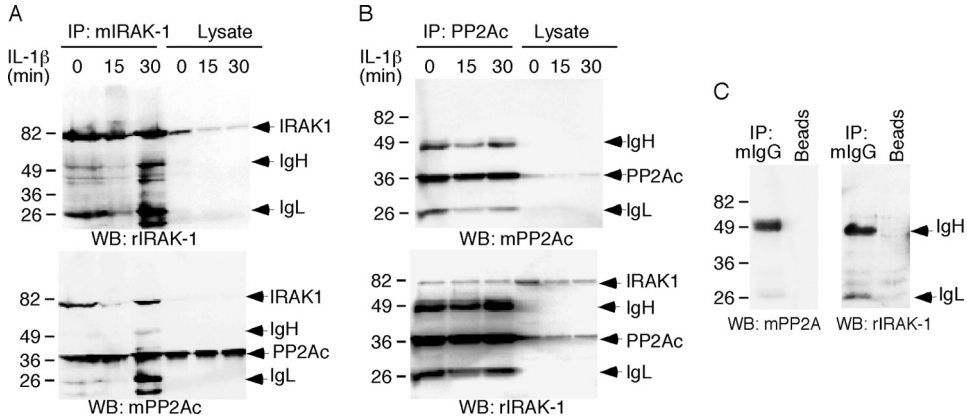

FIGURE 4. **Co-immunoprecipitation and co-localization of endogenous PP2Ac and IRAK-1.** 293-IL-1RI cells were stimulated or not with IL-1β (25 ng/ml) for the indicated times, and IP was done with mouse anti-IRAK-1 (*A*) or mouse anti-PP2Ac (*B*) antibodies or with mouse IgG or agarose beads alone (*C*). The efficiency of immunoprecipitation was monitored by Western blotting (*WB*) using antibodies against IRAK-1 (*A*, *top*) and PP2Ac (*B*, *top*). The interaction with binding partners was visualized after reprobing the membranes with the respective antibodies, anti-PP2Ac (*A*, *bottom*) and anti-IRAK-1 (*B*, *bottom*). Data are representative of four experiments.

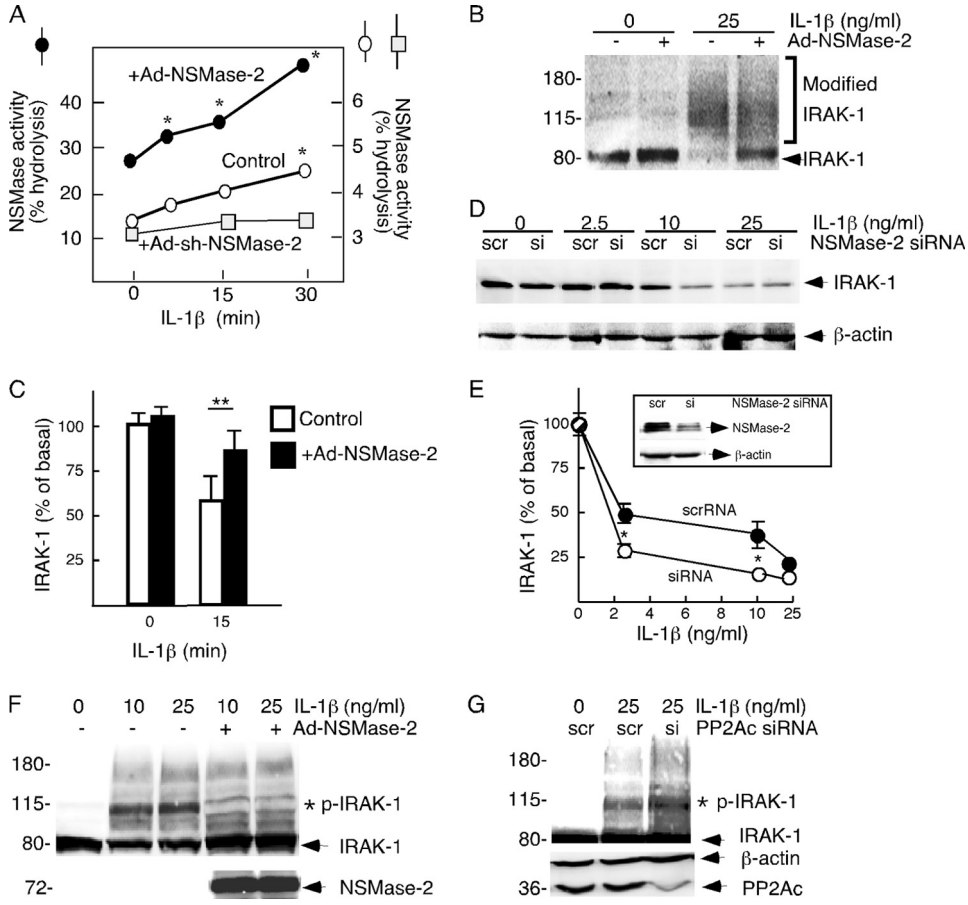

FIGURE 5. **IL-1β-induced activation of NSMase-2 affects IRAK-1 stability.** Primary hepatocytes (*A–E*) or 293-IL-1RI cells (*F* and *G*) were treated with rat or human IL-1β (25 ng/ml) for 30 min, unless indicated otherwise. *A*, hepatocytes were infected with adenovirus encoding mouse NSMase-2 under doxycycline-inducible promoter (*Ad-NSMase-2*), and the expression of NSMase-2 was either induced with doxycycline (*filled symbols*) or not induced (*open symbols*) or they were infected with adenovirus encoding either shRNA against NSMase-2 (*Ad-sh-NSMase-2*; *gray symbols*) or scrambled control (not shown). NSMase activity was measured using C_6-NBD-SM as a substrate. Results are expressed as specific activity (pmol/mg of protein/min). The S.D. value in the assays was within 5% of the measured value ($n = 3$ replicates/assay). *, $p \leq 0.05$. *B–E*, hepatocytes were infected with Ad-NSMase-2 and the expression of NSMase-2 was either induced with doxycycline (*black bars*) or not induced (*white bars*), or they were transfected with either siRNA against the rat NSMase-2 (*open circles*) or scrambled control (*filled circles*). Levels of IRAK-1 were monitored by Western blotting (*B* and *D*) and quantified (*C* and *E*). Results are expressed as percentages of the IRAK-1 levels in untreated hepatocytes. Values are means ± S.D., $n = 3$. *, $p \leq 0.05$; **, $p \leq 0.01$. *Inset*, efficiency of NSMase-2 silencing was determined after simultaneous infection with Ad-NSMase-2. *F* and *G*, 293-IL-1RI cells were infected with Ad-NSMase-2 and the expression of NSMase-2 was either induced with doxycycline or not induced, or they were transfected with either siRNA (*si*) against PP2Ac or scrambled control (*scr*). The levels of IRAK-1, PP2Ac, and NSMase-2 were determined by Western blotting using anti-IRAK-1, anti-PP2Ac, and anti-FLAG antibodies. β-Actin was used as a loading control. Data are representative of at least three independent experiments.

phosphorylation/ubiquitination status is regulated by NSMase-2/PP2A.

Increases in NSMase-2 activity were sufficient to activate PP2A. The basal activity of PP2A was higher in cells overexpressing NSMase-2 by ~250 pmol/min/mg (Fig. 6*A*). IL-1β treatment also was more effective in inducing PP2A activity in NSMase-2-overexpressing cells, where PP2A activation reached 1200 pmol/min/mg as compared with 600 pmol/min/mg in the controls (at 25 ng/ml of IL-1β). OkAc had no effect on cellular NSMase activity (data not shown), indicating that PP2A is downstream of NSMase-2. Accordingly, inhibition of PP2A severely suppressed the effects of NSMase-2 on IRAK-1 stability (Fig. 6*B*).

Ceramide Regulates IRAK-1 Subcellular Localization—To understand further the role of NSMase-2/ceramide/PP2A in IL-1β signaling, IRAK-1 subcellular distribution was examined by indirect immunofluorescence (Fig. 7). In non-stimulated cells, IRAK-1 was observed mainly in the cytosol with some fluorescence seemingly associated with the plasma membrane. IL-1β-stimulated cells exhibited substantial loss in IRAK-1 immunoreactivity, which was in agreement with the reduction in IRAK-1 protein seen on Western blots. This pattern was substantially altered when the IL-1β stimulation was done in the presence of MG-132, ceramide, or bSMase. MG-132 seemed to increase IRAK-1-associated immunofluorescence in an intracellular location as well as on the plasma membrane. Keeping in mind that non-modified IRAK-1, which was elevated by MG-132 (*cf.* Fig. 1), has a very high affinity for binding to the IL-1R·MyD88·IRAK-4 complexes, the increases in plasma membrane IRAK-1 fluorescence may represent non-modified IRAK-1 molecules that reassociated with the receptor complex. Notably, enrichment of the plasma membranes with ceramide by treatment with either bSMase (0.1 unit/ml) or *N*-acetylsphingosine (30 μM) had a similar effect. Together, these results suggest that suppression of IRAK-1 degradation may result in its retention at the

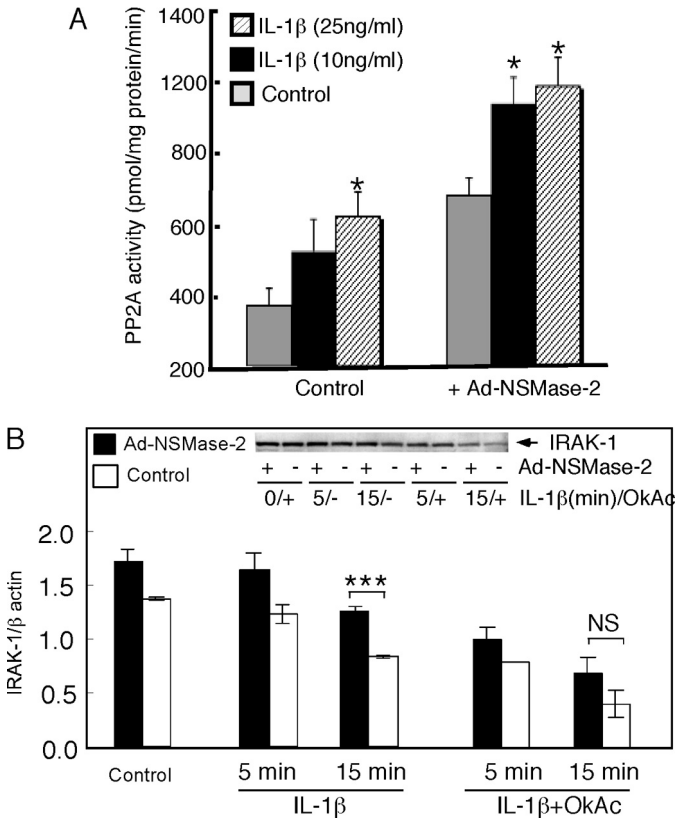

FIGURE 6. **Effects of NSMase-2 on IRAK-1 modification are PP2A-dependent.** 293-IL-1RI cells (*A*) or primary hepatocytes (*B*) were treated with human or rat IL-1β (25 ng/ml) for 30 min, unless indicated otherwise. *A*, 293-IL-1RI cells were infected with Ad-NSMase-2 and induced or not with doxycycline. Phosphatase activity was measured using ^{32}P-MyBP as an exogenous substrate in the presence and absence of 10 nM OkAc. OkAc-sensitive activity, which accounted for 30–40% of the total activity, was attributed to PP2A and expressed in pmol/mg of cell protein/min. Values are means ± S.D. ($n = 3$) and are representative of four different cultures. *, $p \leq 0.05$. *B*, hepatocytes were infected with Ad-NSMase-2, and the expression of NSMase-2 was induced with doxycycline (*black bars*) or not induced (*white bars*). An inhibitor of PP2A, OkAc (10 nM), was added 30 min before stimulation with IL-1β. Levels of IRAK-1 were determined by Western blotting (*inset*) and quantified. β-Actin was used as a loading control. Values are means ± S.D., $n = 3$. ***, $p \leq 0.005$. Data are representative of two independent experiments.

plasma membrane and possible reassociation with active receptor complexes.

NSMase-2 and PP2A Are Part of Positive Feedback Mechanisms of Regulation of IL-1β Response—Stabilization of IRAK-1 has been linked to up-regulation of JNK phosphorylation in the context of LPS signaling (26). Therefore, the effects of NSMase-2 and PP2A on the JNK activation patterns were studied. Inhibition of proteasomal functions in primary hepatocytes as well as overexpression of NSMase-2, both of which are linked to stabilization of IRAK-1, resulted in an increased JNK phosphorylation (Fig. 8, *A–C*). These effects were all suppressed in the presence of OkAc (Fig. 8, *A*, *B*, *D*, and *E*). *Vice versa*, the magnitude of JNK phosphorylation was strongly diminished (Fig. 8*F*) in cells with a low level of NSMase-2 brought about through silencing of NSMase-2 mRNA expression. Together, these data provide compelling evidence that at least in respect to JNK activation, regulation of IRAK-1 degradation can modulate the magnitude of IL-1β response.

DISCUSSION

It has been well known that during IL-1β signaling, IRAK-1 undergoes phosphorylation, Lys^{48} ubiquitination, and degradation in the proteasomes. This study provides evidence that IRAK-1 degradation is dependent not only upon the rate of IRAK-1 phosphorylation but also dephosphorylation. The IRAK-1 dephosphorylation can be stimulated during IL-1β signaling by a mechanism involving NSMase-2 and PP2A, and it is required for maximum activation of JNK. Thus, NSMase-2/ceramide/PP2A form a novel positive feedback regulatory loop for fine tuning of the IL-1β response.

The role of PP2A in the pathogenesis of inflammation has been extensively studied. Traditionally, the phosphatases are considered negative regulators of many cytokine responses (65) because they counteract the phosphorylation of key molecules in the respective pathways. For example, PP2A has been shown to dephosphorylate certain G-protein-coupled receptors, such as the β_2-adrenergic receptor (66) and chemokine receptor CXCR2 (67), thus inhibiting the response at the level of receptor activation. PP2A also directly dephosphorylates transcription factor RelA (55), IκB kinase (IKKβ) (56), and certain MAPK (57), inhibiting their activities. During TNFα response in particular, PP2A dephosphorylates the T loop serines in IKKβ, which results in the inhibition of IKKβ degradation and suppression of NF-κB activation (56). PP2A has also been found to suppress the phosphorylation of MEKK-1, MEKK4, and JNK in response to stimulation with LPS (57).

Our studies provide evidence that in the IL-1β signaling cascade, PP2A participates as a positive effector molecule, and its activation is linked to enhanced inflammatory response. The catalytic activity of PP2A, but not the interaction with its putative substrate, IRAK-1, is regulated. An agonist-independent association of PP2A and its substrates have been established for other substrates, including IKKβ kinase (56) and RelA (55). The existence of these constitutive complexes has been suggested to enhance substrate recognition and to allow for rapid signal-dependent changes in the substrate phosphorylation status. In turn, the mechanisms regulating PP2Ac catalytic activity are not well understood. In the case of IL-1β, stimulation of PP2A catalytic activity is achieved through NSMase-2-dependent generation of ceramide. The activity of NSMase-2 and the levels of ceramide increase 30–45 min following IL-1β stimulation. This time course is in excellent agreement with the time for maximum activation of PP2A. Ceramide is known to directly bind and activate the catalytic subunit of PP2A (47, 61, 64). Alternatively, ceramide is also found to interact with inhibitor 2 of PP2A, causing dissociation of the inhibitor from PP2Ac and release of its inhibitory effect (50).

The interaction of IRAK-1 with PP2A appears to be specific and functional because modulation of PP2A activity by pharmacological inhibitors (okadaic acid), gene silencing, or activating agents (NSMase-2) has a profound effect on IRAK-1 phosphorylation and degradation. However, understanding which phosphorylated residues of IRAK-1 are dephosphorylated by PP2A is difficult at this time because IRAK-1 phosphorylation and ubiquitination are poorly understood. IRAK-1 is first phosphorylated at Thr^{209} by IRAK-4 and then at Thr^{387} and Ser^{376}

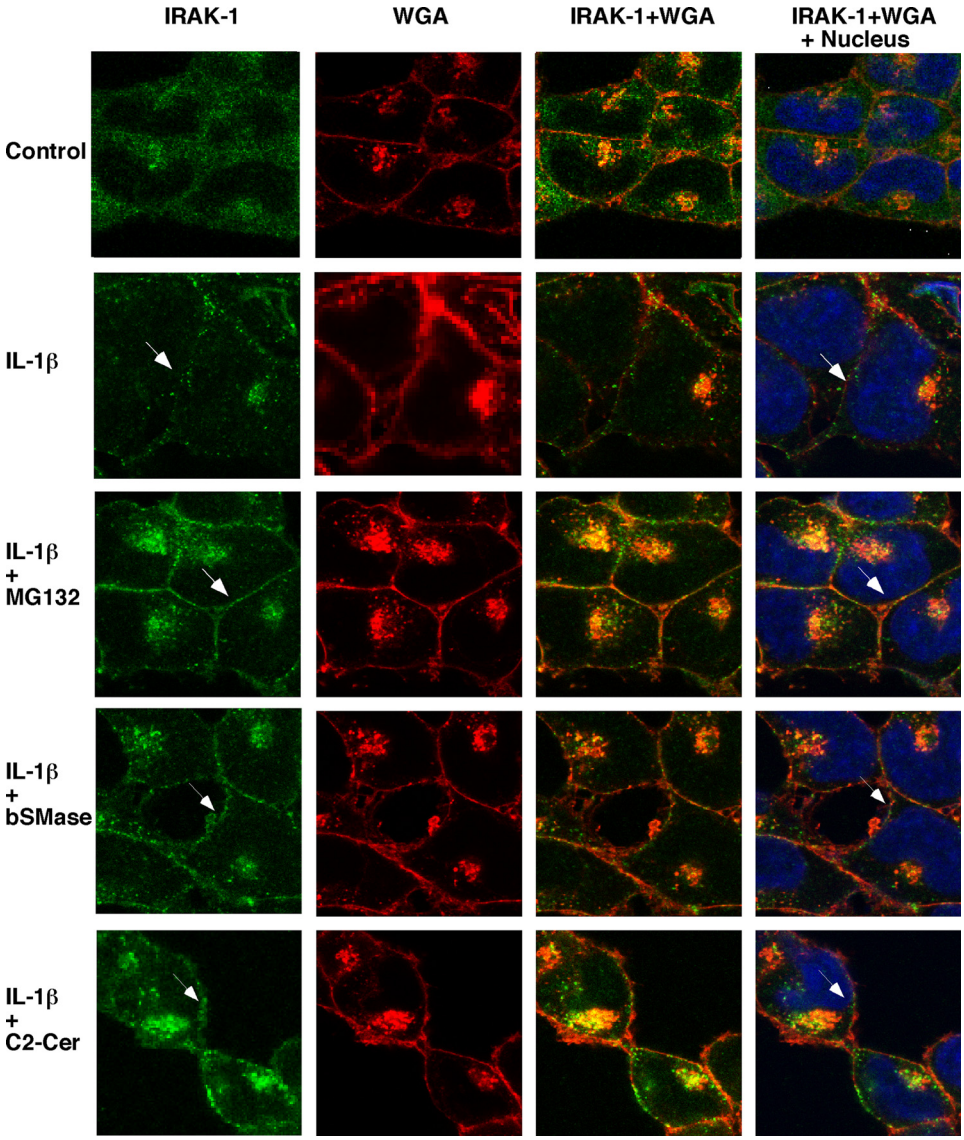

FIGURE 7. **Ceramide regulates IRAK-1 subcellular localization.** 293-IL-1RI cells were treated with MG-132 (50 μM), bSMase (0.1 unit/ml) or *N*-acetylsphingosine (*C2-Cer*) (30 μM) for 30 min and then stimulated with IL-1β (25 ng/ml) for an additional 30 min. The IRAK-1 (*green*) was visualized using antibodies against anti-IRAK1 and confocal microscopy. Wheat germ agglutinin (*WGA*; *red*) and Hoechst (*blue*) were used to visualize plasma membrane and nucleus, respectively.

by both IRAK-4 and itself. This serves to fully induce the kinase activity of IRAK-1, leading to autophosphorylation at additional C-terminal residues in the ProST region (6). This is followed by rapid ubiquitination (16, 19, 24) at several lysine residues in the ProST region and in the kinase domain of IRAK-1 (16–18). Recent studies indicate that, depending on the type of polyubiquitin linkage, IRAK-1 ubiquitination serves diverse roles in downstream signaling. Lys^{63}-mediated ubiquitination promotes protein-protein interaction and downstream signaling, whereas Lys^{48} ubiquitination targets the substrate to the proteasomes for degradation. TRAF6-catalyzed Lys^{63}-linked polyubiquitination at IRAK-1 Lys^{134} and Lys^{180} promotes NF-κB activation because it creates a platform for interaction with NF-κB essential modulator (NEMO), the regulatory subunit of IκB kinase (17). The same residue of IRAK-1 (Lys^{134}) also can undergo Lys^{48}-linked polyubiquitination in a seemingly competitive manner (16). In contrast, Lys^{355} and Lys^{397} of IRAK-1 are modified via Lys^{48}-linked polyubiquitination only and mediate IRAK-1 degradation (18). At the moment, the ubiquitin ligase E3 responsible for the IL-1β-induced Lys^{48}-linked polyubiquitination and degradation of IRAK-1 is unknown.

Consistent with earlier data (16, 24), we found a rapid degradation of IRAK-1 within 30–45 min after IL-1β stimulation in both primary hepatocytes and 293-IL-1RI. Our data consistently show that suppression of the rate of IRAK-1 degradation by various independent approaches (*i.e.* inhibition of proteasomes or NSMase-2 overexpression) results in potentiation of JNK phosphorylation and activity, supporting the notion that IRAK-1 degradation is an important brake in the IL-1β signaling cascade (16). Our indirect immunofluorescence studies, however, also suggest that inhibition of the degradation of IRAK-1 leads to its retention at the plasma membrane, where interaction with active receptor complexes is possible. Furthermore, because MyD88 has high affinity for binding the nonphosphorylated or less phosphorylated IRAK-1 molecules (3, 7)

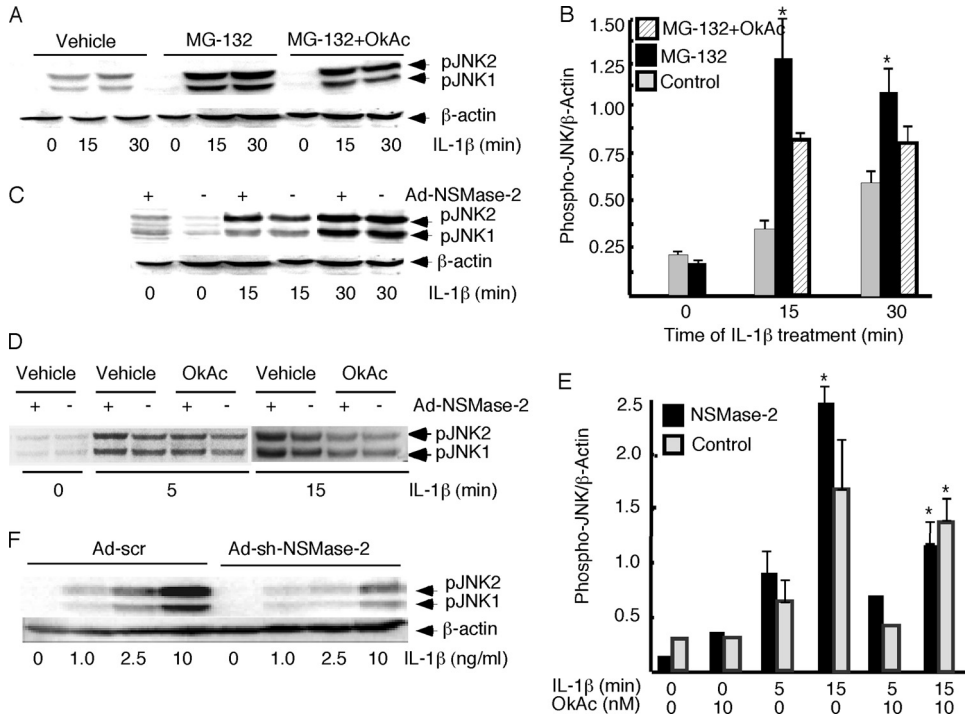

FIGURE 8. **NSMase-2 modulates JNK phosphorylation in a PP2A-dependent manner.** Primary rat hepatocytes were treated with IL-1β (25 ng/ml) for 30 min unless indicated otherwise. *A* and *B*, cells were treated with MG-132 (50 μM) in the presence or absence of OkAc (10 nM) or vehicles for 30 min before the addition of IL-1β. *C–E*, cells were infected with Ad-NSMase-2 and NSMase-2 expression was induced or not with doxycycline. In some experiments (*D* and *E*) OkAc (10 nM) or vehicles were added 30 min before the addition of IL-1β. *F*, hepatocytes were infected with adenovirus encoding shRNA against NSMase-2 (*Ad-sh-NSMase-2*) or scrambled sequence (*Ad-scr*). Levels of JNK phosphorylation were determined by Western blotting using antibodies specific for dually phosphorylated forms of JNK. The combined intensity of bands corresponding to JNK1 and JNK2 was used for quantification. Values are means ± S.D., $n = 3$, *, $p \leq 0.05$. β-Actin was used as a loading control. Data are representative of two independent experiments.

reformation of active MyD88·IRAK-1·TAK-1 complexes is possible, leading to further downstream signaling.

The activation of NSMase and accumulation of ceramide in response to IL-1β stimulation has been well known (28, 32, 68). The form of NSMase that is involved as well as its role in the signaling cascade have began to emerge only recently with the identification of NSMase-2 as the *bona fide* signaling neutral sphingomyelinase (32, 33, 69, 70). In primary hepatocytes (32) and growth-arrested cell lines (34), NSMase-2 is localized at the plasma membrane and is activated by IL-1β and TNFα. In oligodendroma-derived cells, a regulated translocation of NSMase-2 to the caveolae supports further the role of this enzyme in signaling (71). The product of NSMase-2, ceramide, participates in various cellular responses to stress inducers like TNFα, γ-interferon, IL-1, UV, Fas antigen, and NGF (47, 68, 72, 73). The addition of exogenous ceramide or the enhancement of cellular levels of ceramide induces cell differentiation, cell cycle arrest, apoptosis, or cell senescence in various cell types through its ability to regulate the activity of protein kinase Cζ, cathepsin D, Akt, MAPKs, or ceramide-activated protein phosphatases (42, 46, 74).

However, the role of NSMase-2 in hepatic response to IL-1β is more complex. Together with our earlier studies, this paper shows that NSMase-2 and ceramide modulate the IL-1β signaling cascade by fine tuning the magnitude of the response through a positive feedback mechanism. Keeping in mind that the magnitude and the time for induction of the MAPKs, including JNK, are critical for defining specific cellular responses, the newly found role of NSMase-2 might help to understand the mechanisms regulating cellular response to IL-1β and perhaps other members of the TIR family. The growing amount of evidence that NSMase-2 activity is independently regulated during states of oxidative stress, aging, and cancer (33, 39, 60, 75) further emphasizes the potential importance of the NSMase-2-dependent regulation of IL-1β signaling cascade in health and disease.

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