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Functional Regulation of MyD88-Activated Interferon Regulatory Factor 5 by K63-Linked Polyubiquitination▽

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Interferon regulatory factor 5 (IRF-5) plays an important role in the innate antiviral and inflammatory response. Specific IRF-5 haplotypes are associated with dysregulated expression of type I interferons and predisposition to autoimmune disorders. IRF-5 is activated by Toll-like receptor 7 (TLR7) and TLR9 via the MyD88 pathway, where it interacts with both MyD88 and the E3 ubiquitin ligase, TRAF6. To understand the role of these interactions in the regulation of IRF-5, we examined the role of ubiquitination and showed that IRF-5 is subjected to TRAF6-mediated K63-linked ubiquitination, which is important for IRF-5 nuclear translocation and target gene regulation. We show that while the murine IRF-5 and human IRF-5 variant 4 (HuIRF-5v4) and HuIRF-5v5 are ubiquitinated, an IRF-5 bone marrow variant mutant containing an internal deletion of 288 nucleotides is not ubiquitinated. Lysine residues at positions 410 and 411 in a putative TRAF6 consensus binding domain of IRF-5 are the targets of K63-linked ubiquitination. Mutagenesis of these two lysines abolished IRF-5 ubiquitination, nuclear translocation, and the IFNA promoter-inducing activity but not the IRF-5–TRAF6 interaction. Finally, we show that IRAK1 associates with IRF-5 and that this interaction precedes and is required for IRF-5 ubiquitination and activation. Thus, our findings offer a new mechanistic insight into IRF-5 gene induction program through hitherto unknown processes of IRF-5 ubiquitination.

Antiviral cytokines and chemokines are activated as an early response to viral infection and play a critical role in both the outcome of the infection and its pathogenicity. Dysregulation of this response can have pathogenic consequences leading to autoimmune diseases.

Two classes of cellular receptors recognize intracellular viral nucleic acids. Toll-like receptors (TLRs), present in the endosomal compartments of immune cells, detect both viral RNA and DNA(3), whereas TLR3 detects double-stranded RNA, a common replication intermediate of both DNA and RNA viruses (4, 26). TLR7 and TLR8 detect single-stranded viral genomic RNA (10), and DNA viruses are recognized by TLR9, which detects the unmethylated CpG regions of viral genomic DNA (21). TLR7 and TLR9 are expressed predominantly in plasmacytoid dendritic cells, while conventional dendritic cells, fibroblasts, and endothelial cells express TLR3. A second class of receptors which also detects double-stranded RNA produced in infected cells is comprised of the ubiquitously expressed cytoplasmic RNA helicases including RIG-I and MDA5 (33). These receptors signal via the caspase activation and recruitment domains. Although recognition of viral nucleic acids is mediated by distinct cellular receptors, which mediate distinct signaling pathways, they all activate latent transcription factors of the NF-κB and interferon (IFN) regulatory factor (IRF) families (3, 15). While the inducible activation of inflammatory cytokines is largely dependent on NF-κB, IRF-3 and IRF-7 play a critical role in the induction of type I IFN genes.

IRF-5 is a related family member that is implicated in the innate inflammatory response, where it has been shown to control induction not only of type I IFN genes but also of inflammatory cytokines and chemokines. IRF-5 controls the transcription of type I IFN genes in certain cell types, while its role in the induction of inflammatory cytokines and chemokines is more general (5, 7, 23). Several distinctly spliced human IRF-5 (HuIRF-5) isoforms (designated variants 1 to 10), which show cell-type-specific expression and distinct cellular localization, have recently been identified (9, 20, 22). The most common variations feature insertions or deletions in exon 6. A common single nucleotide polymorphism haplotype in IRF-5 was identified as a risk factor for systemic lupus erythematosus (SLE) (12, 13), an autoimmune disease characterized by the presence of excess type I IFN in the serum. Thus, the dysregulated expression of type I IFNs, which is associated with SLE, may be related to the presence of elevated levels of SLE-promoting IRF-5 haplotypes.

Activation of IRF-5 is restricted to TLRs that signal via MyD88, including TLR4, TLR5, TLR7, TLR8, and TLR9 (25). MyD88-dependent activation of IRF-5 involves formation of a tertiary complex consisting of interleukin-1-associated kinase 1 (IRAK1), IRAK4, tumor necrosis factor receptor-associated factor 6 (TRAF6) together with IRF-5 or IRF-7 (18, 28). Mechanistically, MyD88 is recruited to the TLRs upon ligand binding, leading to the activation of IRAK1 and IRAK4, which then associate with TRAF6. TRAF6 is a K63-specific ubiquitin ligase that forms complexes exclusively with the UBC13 and Uev1a/Mms2 heterodimer acting as an E2, which then promotes synthesis of lysine 63-linked polyubiquitin chains (31). Polyubiquitination through K63 does not generally target proteins for proteasomal degradation but regulates important cel-

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lular process such as DNA repair (30) and signal transduction (1). It was shown recently that in both TLR and nucleotide oligomerization domain signaling pathways, TRAF6 mediates K63-linked ubiquitination of NEMO (1) and increases activation of NF-κB. Furthermore, it has been shown that, in response to TLR9 ligands, TRAF6 binds to and ubiquitinates IRF-7 and that the E3 ligase activity of TRAF6 is required for IRF-7 ubiquitination. However, neither the functional role of IRF-7 ubiquitination nor its mechanism was established (18).

The aim of this study was to examine the molecular mechanism of IRF-5 activation by the MyD88-dependent TLR pathway, focusing on the role of TRAF6-dependent K63-linked ubiquitination of IRF-5 activation. We demonstrate that in MyD88 signaling, IRF-5 associates with MyD88 and undergoes K63-linked ubiquitination, translocation to the nucleus, and association with the promoters of type I IFN genes. K63 ubiquitination via TRAF6 targets lysines at positions 410 and 411, which are located in the TRAF6 binding motif of IRF-5. The mutations of lysine to arginine residues at these sites leads to an accumulation of IRF-5 in the cytoplasm, resulting in abrogation of IRF-5-mediated stimulation of the IFNA promoter. Finally, we show that IRF-5 interacts with IRAK1 and that TRAF6 ubiquitination of IRF-5 is dependent on IRAK1 since this was not detected in MyD88-stimulated IRAK1$^{-/-}$ cells. These results show that association of MyD88 with IRAK1 activates IRF-5 and that these events precede the TRAF6-dependent ubiquitination of IRF-5, a critical event in the regulation of IRF-5 function.

MATERIALS AND METHODS

Expression plasmids. pcDNA6 Myc/His-tagged IRF-5 was described previously (23); hemagglutinin (HA)-tagged wild-type ubiquitin was a generous gift from H. Gottlinger (University of Massachussetts Medical School, Worcester, MA), and HA-tagged K63R and HA-tagged K48R were generously provided by Z. Chen (University of Texas Southwestern Medical Center, Dallas, TX). The IRF-5 K410R K411R (K410/K411R) point mutant was generated from pcDNA6-IRF-5 through a QuikChange site-directed mutagenesis kit (Stratagene). The IRF-5 variant 4 (IRF-5v4) C-terminal deletions were described previously (6). The IRF-5v5 mutants containing C-terminal deletions after the TRAF6 consensus binding site PREKKL was generated by PCR. The amplification step was performed with *Pfu* Turbo DNA polymerase (Stratagene). The BamHI and EcoRI restriction sites were included in the primer design. The amplified fragments were gel purified and cloned in-frame within the BamHI and EcoRI restriction sites in the pCMV-3Tag vector-1 (catalogue no. 240195; Stratagene). The sequence was confirmed by sequencing. The MyD88, TRAF6, IRAK1, and IRAK1 D340N expression plasmids were described previously (11). Luciferase reporter plasmids containing promoters of murine IFNA and IFNB genes were a generous gift from T. Michiels (University of Louvain, Belgium).

Cell lines and antibodies. 293T cells were maintained in Dulbecco's modified Eagle's medium with 5% fetal bovine serum (FBS). TRAF6$^{-/-}$ mouse embryonic fibroblasts (MEFs) were kindly provided by Tak Mak (University of Toronto, Toronto, Canada) and 293TI1A IRAK1-deficient cells were a kind gift from X. Li (Cleveland Clinic, Cleveland, OH) and were maintained in Dulbecco's modified Eagle's medium supplemented with 10% FBS and 2 mM glutamine. RAW264.7 cells were cultured in RPMI medium supplemented with 10% FBS and stimulated with 100 ng/ml R848 for 8 h.

Polyclonal antibodies, rabbit MyD88, β-actin, α-tubulin, Sp-1 antibodies, and monoclonal IRF-5 antibodies were purchased from Santa Cruz Biotechnology; anti-HA antibody was purchased from (Roche), and antiubiquitin polyclonal antibodies were purchased from Sigma. Mouse anti-His antibodies were from Zymed Laboratories. Anti-mouse IRF-5 antibodies were generated in rabbits against the VRFPSPEDIPSDKWR peptide by Affinity BioReagents. These antibodies cross-react with HuIRF-5. The M2 anti-Flag beads were purchased from Sigma. Antiubiquitin-Sepharose beads were purchased from Pierce Biotech.

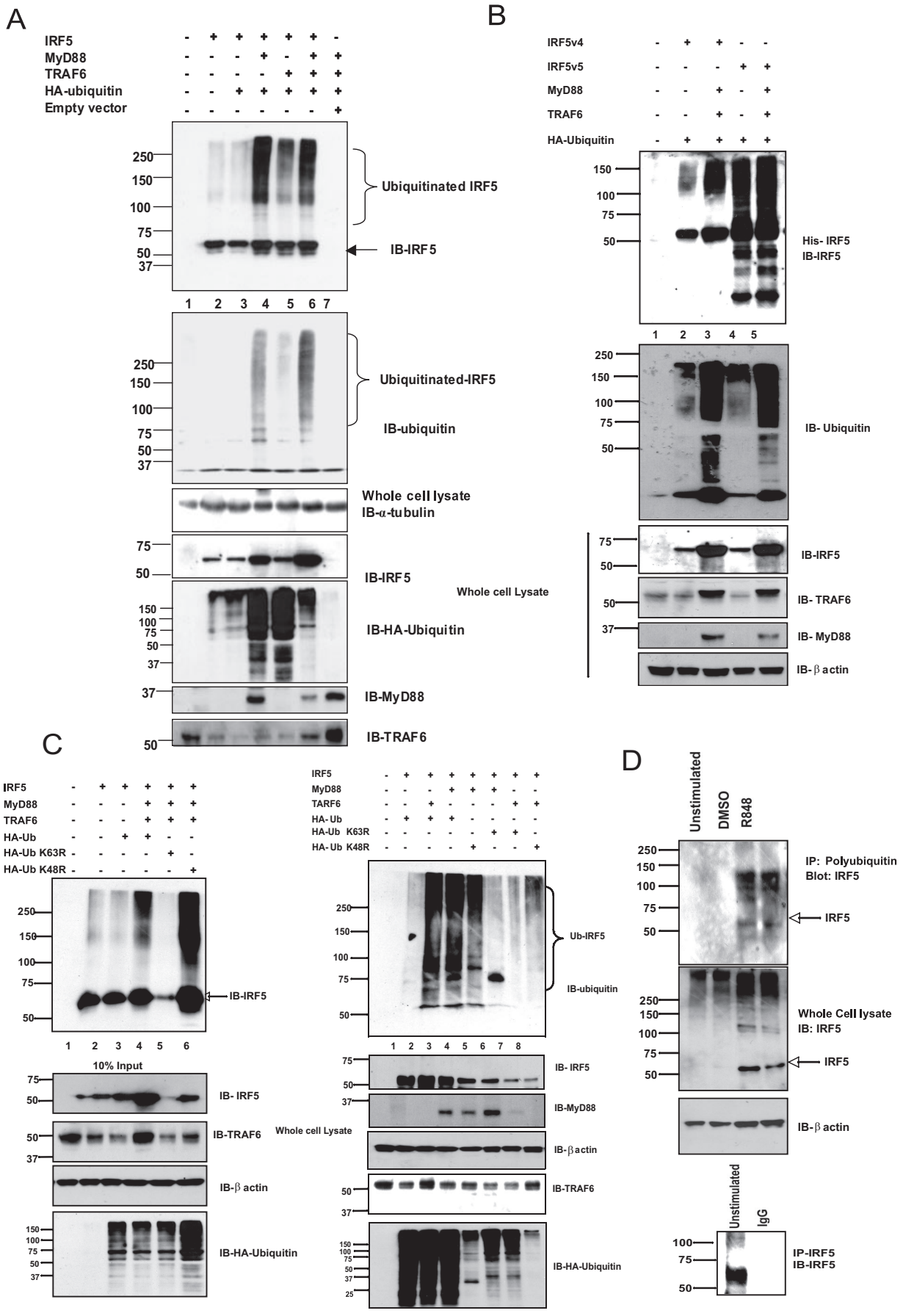
Subcellular localization and chromatin immunoprecipitation (ChIP). For preparation of the cytoplasmic extracts, cells were lysed in a buffer containing 20 mM Tris (pH 8), 10 mM NaCl, 3 mM $MgCl_2$, 20% glycerol, 0.2 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1% NP-40. Nuclear extracts were prepared from 2×10^7 293T cells that were lysed in a buffer containing 20 mM Tris (pH 8), 400 mM NaCl, 20% glycerol, 0.2 mM EDTA, 1 mM DTT, and protease and phosphatase inhibitor cocktail (Sigma).

For the ChIP analysis, 293T cells (2×10^7) were cotransfected with IRF-5 and a combination of MyD88, TRAF6, and ubiquitin-expressing plasmids (1 μg of each). At 24 h posttransfection the ChIP assay was done as described previously (6). Briefly, proteins bound to DNA were cross-linked by the addition of formaldehyde to a final concentration of 1% for 30 min at 37°C, and the cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. Cells were then washed twice in ice-cold phosphate-buffered saline containing 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, and 1 μg/ml pepstatin; cells were pelleted, resuspended in 200 μl of sodium dodecyl sulfate (SDS) lysis buffer, and sonicated for 1 min. Samples were precleared with protein A-agarose beads, and the input DNA levels were measured in 1% of the total precleared sample. Samples were then immunoprecipitated with polyclonal anti-IRF-5 (2 μg) or immunoglobulin G antibody (Affinity BioReagents). Immune complexes were washed and resuspended in Tris-EDTA buffer prior to treatment with RNase A (50 μg/ml), 0.5% SDS, and proteinase K (500 μg/ml). Cross-linking was reversed by heating to 65°C for 6 h, and DNA was recovered by phenol-chloroform extraction and purified by precipitation with 2 M ammonium acetate-ethanol. DNA was resuspended in 30 μl of water, and 10 μl was used for PCR amplification with IFNA4, IFNA13, and IFNB promoter-specific primers for 30 cycles.

Affinity purification of polyhistidine-tagged IRF-5. Cells (5×10^7) were cotransfected with IRF-5 and a combination of expression plasmids for 24 h. Cells were then harvested and lysed under denaturating conditions using guanidium lysis buffer (pH 7.8). Lysates were then passed through an 18-gauge needle and centrifuged to remove cellular debris. Ni^+-charged ProBond resin beads (catalogue no. K850-01; Invitrogen) were preblocked by incubation with bovine serum albumin and washed with binding buffer before incubation with cell lysates for 1 h. Beads were collected by centrifugation at $800 \times g$ for 1 h and washed with native purification buffers with enhanced stringency (250 mM NaH_2PO_4, pH 8.0, 2.5 M NaCl, 3 M imidazole, protease and phosphatase inhibitor cocktails). Beads were then washed with phosphate-buffered saline, resuspended in 1× loading buffer, and heated to 95°C for 5 min, and bound proteins resolved by SDS-polyacrylamide gel electrophoresis (PAGE). IRF-5 and associated proteins were detected by immunoblotting with specific antibodies.

Transfections, immunoprecipitation, and immunoblot analysis. For the transient transfection assays, 2×10^6 cells were transfected with various expression plasmids by using Polyfect transfection reagent (Qiagen). Empty vector DNA was used to keep the total amount of transfected DNA constant (total, 4 μg). Samples were lysed in a radioimmunoprecipitation assay lysis buffer (20 mM Tris [pH 7.9], 50 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.2 mM protease inhibitor mixture; Sigma). For SDS-PAGE, 10 to 20 μg of total protein was resolved on 8 or 10% polyacrylamide gels and transferred to nitrocellulose or polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature with 5% dry skim milk powder in Tris-buffered saline–Tween 20 before overnight incubation with the respective antibodies. The membranes were subsequently incubated for 1 h at room temperature with horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin G antibody (Amersham Biosciences), and immunodetection was visualized by ECL reagents (Amersham), followed by autoradiography on HyBlot CL film (Denville Scientific). Membranes were stripped of bound antibodies using a stripping solution (Pierce). For Flag immunoprecipitation, anti-Flag affinity gel (Sigma A2220) was used according to the vendor's directions.

Luciferase reporter assay. 293T cells and IRAK1$^{-/-}$ and TRAF6$^{-/-}$ MEF cells seeded on 24-well plates (1×10^5) were transfected with 100 ng of the luciferase reporter plasmid together with a total of 400 ng of various expression plasmids using Lipofectamine 2000 (Invitrogen). The total amounts of transfected DNA were kept constant in all experiments by adjustment with empty vector. Luciferase activity was measured 24 h later using a dual luciferase reporter assay system (Promega). The *Renilla* luciferase gene (20 ng) was cotransfected and was used as an internal control plasmid. Each experiment was repeated four times. Data are expressed as means ± standard deviations of four replicates.


A
IRF5
MyD88
TRAF6
HA-ubiquitin
Empty vector
Ubiquitinated IRF5
IB-IRF5
Ubiquitinated-IRF5
IB-ubiquitin
Whole cell lysate
IB-α-tubulin
IB-IRF5
Whole cell Lysate
IB-HA-Ubiquitin
IB-MyD88
IB-TRAF6
B
IRF5v4
IRF5v5
MyD88
TRAF6
HA-Ubiquitin
His- IRF5
IB-IRF5
IB- Ubiquitin
IB-IRF5
IB- TRAF6
IB- MyD88
IB- β actin
C
IRF5
MyD88
TRAF6
HA-Ub
HA-Ub K63R
HA-Ub K48R
IB-IRF5
10% Input
IB- IRF5
IB-TRAF6
IB- β actin
IB-HA-Ubiquitin
TARF6
Ub-IRF5
IB-ubiquitin
IB- IRF5
IB-MyD88
Whole Cell Lysate
IB- β actin
IB-TRAF6
IB-HA-Ubiquitin
D
Unstimulated
DMSO
R848
IP: Polyubiquitin
Blot: IRF5
IRF5
Whole Cell lysate
IB: IRF5
IRF5
IB- β actin
Unstimulated
IgG
IP-IRF5
IB-IRF5

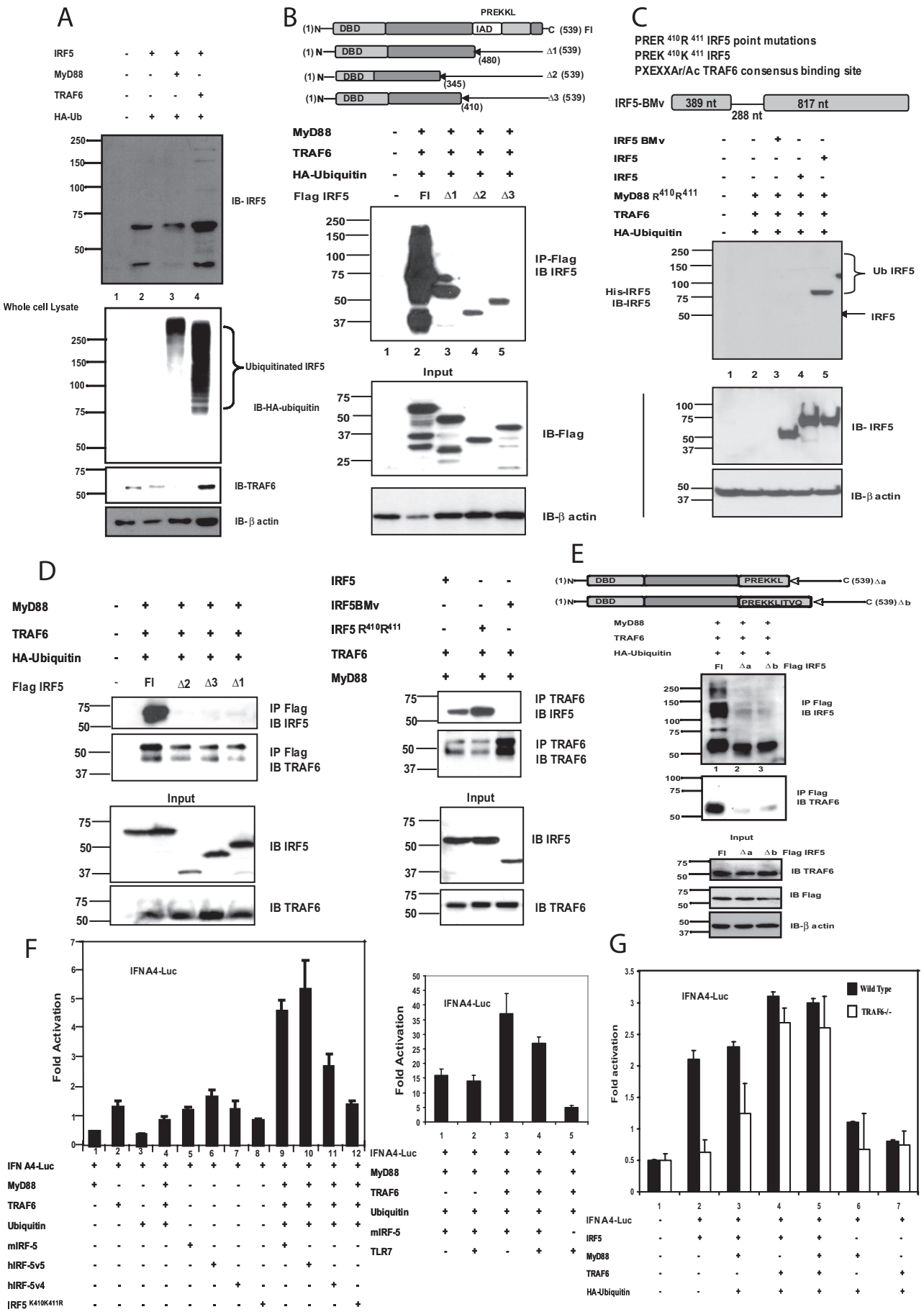
RESULTS

IRF-5 undergoes K63-linked polyubiquitination in response to TLR/MyD88-dependent signaling. The observation that IRF-5 associates with TRAF-6 in the TLR-MyD88 signaling pathway (28) prompted us to investigate whether the association of TRAF6 with IRF-5 results in the ubiquitination of IRF-5. To this effect, cells were transfected with murine IRF-5 (referred to here as IRF-5) and various combinations of MyD88, TRAF6, and HA-tagged ubiquitin expression plasmids. At 24 h after transfection cells were lysed, and His-tagged IRF-5 was purified by affinity purification on a Ni column. The results clearly show that MyD88-activated IRF-5 underwent a robust ubiquitination in cells expressing ectopic ubiquitin and TRAF6 (Fig. 1A, lanes 4 and 6). The activation of IRF-5 by MyD88 was necessary for this ubiquitination since in the absence of MyD88 the ubiquitination of IRF-5 was significantly weaker. When cells were expressing IRF-5 alone or IRF-5 cotransfected with ubiquitin plasmid alone, IRF-5 was not ubiquitinated (Fig. 1A, lanes 2 and 3). These data show that IRF-5 undergoes ubiquitination when activated by MyD88. We also examined the ubiquitination of human isoforms of IRF-5, notably IRF-5 v4 and IRF-5 v5. Results show that the Ni-purified HuIRF-5 variants undergo MyD88-TRAF6-dependent ubiquitination (Fig. 1B, lanes 3 and 5). The expression of IRF-5 variants, MyD88, and TRAF6 was detected after immunoblotting from the input whole-cell lysate (Fig. 1B). IRF-5 ubiquitination could also be detected in cell lysates of 293T cells by immunoblotting when the cells were transfected with IRF-5 in the presence of MyD88 but not in its absence, showing that MyD88-mediated activation of IRF-5 enhances its ubiquitination.

TRAF6-mediated ubiquitination occurs predominantly through K63-linked, rather than through K48-linked, chains. A K63-linked modification does not target proteins for degradation but leads to activation of signaling functions. To determine whether ubiquitination of IRF-5 was K63 or K48 linked, we used two ubiquitin mutant plasmids, in which residues 63 or 48 were mutated to arginine, i.e., K63R and K48R, respectively. 293T cells were transfected with IRF-5 together with MyD88, TRAF6, wild-type ubiquitin, and K63R and K48R HA-tagged ubiquitin mutants (Fig. 1C). Cells were harvested 24 h posttransfection, and IRF-5 was purified on a Ni column, analyzed by SDS-PAGE, and detected by immunoblotting with IRF-5 (Fig. 1C, left panel) or ubiquitin-specific antibodies (right panel). The results revealed that only the wild-type ubiquitin and the K48R mutants mediated ubiquitination of IRF-5 (Fig. 1C, left panel, lane 6, and right panel, lane 8) while no ubiquitination of IRF-5 was detected when the K63R mutant was examined (left panel, lane 5, and right panel, lanes 6 and 7). These results show that ubiquitination of IRF-5 is K63 linked.

We also examined whether engagement of TLRs leads to the ubiquitination of endogenous IRF-5. To this effect, we used the RAW264.7 macrophage cell line, which expresses TLR7 and responds to the TLR7 ligand R848 to activate both IRF-7 and IRF-5 (17, 25). Cells were mock treated (with dimethyl sulfoxide [DMSO]) or stimulated with R848 for 8 h or left untreated. The polyubiquitinated proteins were enriched on an antiubiquitin column and then subjected to immunoblotting with anti-IRF-5 antibody. As a positive control, RAW264.7 cells were also cotransfected with IRF-5, MyD88, TRAF6, and ubiquitin expression plasmids. A significant enrichment of ubiquitinated IRF-5 was detected in R848-treated cells and in cells transfected with MyD88, TRAF6, and ubiquitin (Fig. 1D). The ubiquitination pattern of the endogenous IRF-5 was similar to that obtained in the cotransfection experiment. No ubiquitinated endogenous IRF-5 was detected in the untreated or DMSO-treated cells. The Western blot analysis of cell lysates of R848-treated and transfected cells also revealed ubiquitinated IRF-5. Interestingly, the levels of expression of IRF-5 in untreated or mock-treated cells were very low, and IRF-5 was detected in total cell lysates only in R848-treated or transfected cells but not in the controls, indicating that TLR signaling upregulated IRF-5, probably via autocrine IFN signaling. However, when enriched by immunoprecipitation, IRF-5 was detected in lysates of untreated cells, but it was not ubiquitinated (Fig. 1D, lower panel).

FIG. 1. IRF-5 undergoes K63-linked polyubiquitination in a MyD88-dependent pathway. (A) 293T cells transfected with a total (4 μg) of His-tagged IRF-5 (lanes 2 to 6), MyD88 (lanes 4, 6, and 7), and TRAF6 (lanes 5, 6, and 7) together with HA-ubiquitin (lanes 3 to 7) IRF-5 were affinity purified from cell lysates, using Probond Ni^{2+}-charged resin. Bound proteins were separated on an SDS gel, and IRF-5 was identified by immunoblotting (IB) with anti-IRF-5 (upper panel) and antiubiquitin antibody (lower panel) after stripping the same blot. Whole-cell lysate was also immunoblotted with tubulin, anti-MyD88, anti-TRAF6, and anti-HA antibodies. (B) 293T cells were transfected with IRF-5v4 or IRF-5v5 and ubiquitin together with MyD88 and TRAF6. At 24 h after transfection cells were lysed, and IRF-5v4 was immunoprecipitated with Flag antibodies. IRF-5v5 was affinity purified on Ni resin. Proteins were separated on an SDS gel and immunoblotted with anti-IRF-5 and antiubiquitin antibody (upper panels). The relative levels of IRF-5, β-actin, and TRAF6 in the whole-cell lysate determined by immunoblotting are shown. (C) 293T cells were transfected with His-tagged IRF-5 (lanes 2 to 6 in the left panel and lanes 2 to 8 in the right panel), MyD88 (lanes 4 to 6 in the left panel and lanes 4 to 6 in the right panel), TRAF6 (lanes 4 to 6 in the left panel and lanes 3, 8, and 9 in the right panel) together with HA-ubiquitin (lanes 3 and 4 in the left panel and lanes 3 and 4 in the right panel) or a K63R HA-ubiquitin mutant (lane 5 in the left panel and lanes 6 and 7 in the right panel) or K48R ubiquitin mutant (lane 6 in the left panel and lanes 7 to 8 in the right panel). His-tagged IRF-5 was affinity purified on Ni resin and blotted with anti-IRF-5 (left panel) or antiubiquitin (right panel) serum. The expression of IRF-5, MyD88, TRAF6, and K48 or K63 HA-ubiquitin detected in the input cell lysates was detected by immune blotting (lower panels). (D) Ubiquitination of the endogenous IRF-5. RAW264.7 cells were either treated with DMSO or stimulated with a 100 nM concentration of TLR7 agonist R848 for 8 h to engage TLR7-MyD88 pathway that leads to IRF-5 activation. As a positive control, cells were transfected with IRF-5 together with MyD88, TRAF6, and ubiquitin. Cells were lysed with radioimmunoprecipitation assay buffer, and ubiquitinated IRF-5 was immunopurified using antiubiquitin beads. The specifically bound proteins were subjected to SDS-PAGE and immunoblotted with IRF-5 antibodies (upper panel). The total cell lysate was probed with IRF-5 (lower panel). The cell lysate from unstimulated RAW264.7 cells was immunoprecipitated with IRF-5 and immunoblotted with IRF-5 to demonstrate the expression of an endogenous IRF-5 (lower panel). Immune blotting with ubiquitin antibodies did not detect any IRF-5 (data not shown). Ub, ubiquitin; IgG, immunoglobulin G.


A
IRF5
MyD88
TRAF6
HA-Ub
IB- IRF5
Whole cell Lysate
Ubiquitinated IRF5
IB-HA-ubiquitin
IB-TRAF6
IB-β actin
B
PREKKL
DBD
IAD
C (539) Fl
Δ1 (539)
(480)
Δ2 (539)
(345)
Δ3 (539)
(410)
MyD88
TRAF6
HA-Ubiquitin
Flag IRF5
Fl
Δ1
Δ2
Δ3
IP-Flag
IB IRF5
Input
IB-Flag
IB-β actin
C
PRER 410R 411 IRF5 point mutations
PREK 410K 411 IRF5
PXEXXAr/Ac TRAF6 consensus binding site
IRF5-BMv
389 nt
288 nt
817 nt
IRF5 BMv
IRF5
MyD88 R410R411
TRAF6
HA-Ubiquitin
His-IRF5
IB-IRF5
Ub IRF5
IRF5
IB- IRF5
IB-β actin
D
MyD88
TRAF6
HA-Ubiquitin
Flag IRF5
Fl
Δ2
Δ3
Δ1
IP Flag
IB IRF5
IP Flag
IB TRAF6
Input
IB IRF5
IB TRAF6
IRF5
IRF5BMv
IRF5 R410R411
TRAF6
MyD88
IP TRAF6
IB IRF5
IP TRAF6
IB TRAF6
Input
IB IRF5
IB TRAF6
E
PREKKL
C (539) Δa
PREKKLITVQ
C (539) Δb
MyD88
TRAF6
HA-Ubiquitin
Fl
Δa
Δb
Flag IRF5
IP Flag
IB IRF5
IP Flag
IB TRAF6
Input
IB TRAF6
IB Flag
IB-β actin
F
IFNA4-Luc
Fold Activation
IFN A4-Luc
MyD88
TRAF6
Ubiquitin
mIRF-5
hIRF-5v5
hIRF-5v4
IRF5 K410K411R
IFNA4-Luc
MyD88
TRAF6
Ubiquitin
mIRF-5
TLR7
G
IFNA4-Luc
Wild Type
TRAF6-/-
Fold activation
IFNA4-Luc
IRF5
MyD88
TRAF6
HA-Ubiquitin

TRAF6 consensus binding motif in IRF-5 is essential for IRF-5 polyubiquitination. TRAF6 is a member of the TRAF family that has been shown to function as an E3 ubiquitin ligase (27). Binding of TRAF6 to IRF-7 or IRF-5 has been demonstrated previously (18, 28). To determine whether the ubiquitination of IRF-5 is mediated by TRAF6 or whether other members of the TRAF family also contribute to IRF-5 ubiquitination in the TLR signaling pathway, we have examined the ubiquitination of IRF-5 in TRAF6$^{-/-}$ MEFs. These cells were cotransfected with IRF-5 and ubiquitin in combination with either MyD88 or TRAF6, and at 24 h posttransfection, IRF-5 was analyzed by immunoblotting. The results showed that MyD88 activation of IRF-5 ubiquitination was compromised in MEFs lacking TRAF6, which was restored upon reconstitution with ectopic TRAF6. (Fig. 2A, lane 4), thus indicating that TRAF6 contributes to K63-linked ubiquitination of IRF-5. A very slow mobility band was observed in HA immunoblots of cellular extracts when IRF-5 and MyD88 were cotransfected into TRAF6-negative cells, indicating the possible involvement of another member of the TRAF family or another cellular E3 ubiquitin ligase in TRAF6-independent IRF-5 polyubiquitination.

We also analyzed ubiquitination of a set of HuIRF-5v4 C-terminal deletion mutants which were tagged with a Flag epitope. A schematic representation of these deletion mutants is shown in Fig. 2B. The mutants were transfected together with ubiquitin, MyD88, and TRAF6 expression plasmids into 293T cells. Western blot analysis of transfected lysates showed that all the mutants were expressed at comparable levels. IRF-5 was next immunoprecipitated and immunoblotted with anti-IRF-5 antibodies. As shown in Fig. 2B, full-length IRF-5 shows several slowly moving bands. These bands were also detected by ubiquitin antibodies, indicating that they represent polyubiquitinated IRF-5 (data not shown). In contrast, none of the deletion mutants was ubiquitinated (Fig. 2B, lanes 3 to 5), and only full-length IRF-5 was ubiquitinated (lane 2). These data indicate that the ubiquitination sites targeted by the TRAF6 pathway are localized in the C′-terminal region of the IRF-5 polypeptide. Sequence analysis of this region identified a putative TRAF6 consensus binding motif [PXEXX(Ar/Ac), where Ar/Ac is an aromatic/acidic residue] (32) near the C′-terminal region of IRF-5 (Fig. 2C). IRF-5 mutants which have the carboxyl-terminal region with a deletion of the TRAF6 recognition site are not ubiquitinated (Fig. 2B). Interestingly, this region also contains two lysine residues. To examine the role of these two lysine residues in IRF-5 ubiquitination, we mutated both lysine K410 and K411 to arginine and tested the ubiquitination of this mutant (K410/K411R). 293T cells were cotransfected with IRF-5 or the IRF-5 K410/K411R mutant together with MyD88, TRAF6, and ubiquitin plasmids. After the Ni affinity purification of IRF-5, samples were immunoblotted with anti-IRF-5 or with anti-ubiquitin antibodies (Fig. 2C). Results shown in Fig. 2C revealed that while IRF-5 underwent a robust ubiquitination in a MyD88-TRAF6-dependent manner, no ubiquitination of the double point mutant K410/K411R was observed (lanes 4). We also examined whether the spliced murine IRF-5 bone marrow variant (IRF-5-BMv) is ubiquitinated; however, no ubiquitination of this mutant was detected (lane 3). The immunoblotting of the transfected lysates showed that all the IRF-5s encoded by the respective plasmids were expressed at equivalent levels. These data indicate that deletion of the TRAF6 recognition site in the IRF-5 peptide or mutation of the lysines 410 and 411 prevents TRAF6–IRF-5 interactions or that the lysines 410

FIG. 2. IRF-5 is polyubiquitinated by the E3 ligase TRAF6. (A) TRAF6$^{-/-}$ MEFs were cotransfected with IRF-5, MyD88, and HA-ubiquitin in the presence and absence of TRAF6. At 24 h posttransfection, cell lysates were immunoblotted with anti-IRF-5 (upper panel) or anti-HA epitope antibodies (middle panel). To verify TRAF6 expression, the blot was reprobed with anti-TRAF6 antibody (lower panel). Levels of β-actin show equal loading of protein. (B) Schematic structure of C-terminal deletions of IRF-5v4. DBD, DNA binding domain; IAD, interacting domain. Amino acid regions that are deleted in the IRF-5 mutants are indicated. Flag-tagged full-length IRF-5 or its deletion mutants were transfected to 293T cells together with MyD88, TRAF6, and ubiquitin. At 24 h posttransfection cells were lysed, and lysates were subjected to immunoprecipitation using anti-Flag-coupled beads followed by immunoblotting with anti IRF-5 antibody. IRF-5 and its deletion mutants were detected in cell lysates by immunoblotting with anti-Flag antibody. (C) A schematic illustration of point mutations at TRAF6 consensus recognition motif and a deletion in IRF-5-BMv. 293T cells were transfected with murine IRF-5 (positive control), IRF-5-BMv, and IRF-5 K410/K411R together with MyD88, TRAF6, and ubiquitin. His-purified IRF-5 was detected by immunoblotting with anti-IRF-5 (upper panel). IRF-5-BMv and IRF-5 mutant expression in cell lysates were detected by immunoblotting, and the relative levels of β-actin indicate equal protein loading. (D) Binding of TRAF6 to IRF-5 and its mutants. Cells were cotransfected with Flag-IRF-5 or its deletion mutants together with MyD88 and HA-ubiquitin expression plasmids (left). Twenty-four hours later the cells were lysed, and the lysates were precipitated with Flag antibodies; the presence of IRF-5 and TRAF6 in the precipitates was detected by Western blotting with IRF-5 or TRAF6 antibodies. The relative levels of transfected IRF-5, its mutants, and TRAF6 in the input lysates were detected by Western blotting. The IRF-5-positive band in the control sample (lane 1) was caused by a leaking well. Lysates from cells cotransfected with IRF-5, the IRF-5 K410/K411R mutant, and IRF-5-BMv with MyD888 and TRAF6 were immunoprecipitated with TRAF6 antibodies, and the levels of IRF-5 and TRAF6 in immunoprecipitated samples were detected by immunoblotting with IRF-5 or TRAF6 antibodies (right panels). The relative levels of transfected IRF-5, the IRF-5 K410/K411R mutant, IRF-5-BMv, and TRAF6 in the input lysates were detected by immunoblotting with respective antibodies. (E) The two C-terminal deletion mutants of IRF-5v5 containing the TRAF6 consensus recognition sequence PREKKL are shown schematically. The lysates of cells cotransfected with full-length IRF-5v5 or its mutants and MyD88 and TRAF6 were immunoprecipitated with Flag antibody, and IRF-5 was detected by immunoblotting with IRF-5 antibody (upper panel). The same blot was stripped of bound antibody and blotted with TRAF6 antibody (middle panel). The levels of expression of IRF-5 and TRAF6 in input lysates are shown in the lower panel. (F) Mutation in the TRAF6 consensus recognition motif of IRF-5 blocked activation of IFNA4 reporter. 293T cells were cotransfected with an IFNA4-luc reporter plasmid (10 ng) together with the indicated combination of MyD88 (10 ng), TRAF6 (10 ng), and ubiquitin (5 ng) and 10 ng each of IRF-5, IRF-5v5, IRF-5v4, and the IRF-5 K410/K411R mutant. Luciferase activity was measured 24 h after the transfections. Cells were also transfected with IRF-5, MyD88, and TRAF6 and together with TLR7 expression plasmid. Sixteen hours after transfections stimulated with 10 nM R848 for 8 h, luciferase activity was measured in cell lysates (right panel). (G) IFNA4-luciferase (Luc) reporter and IRF-5 plasmids were cotransfected into TRAF6$^{-/-}$ MEFs and wild-type MEFs together with MyD88, TRAF6, and ubiquitin as indicated. The levels of DNA were kept constant in all transfection experiments, and the data are expressed as means ± standard deviations of four replicates. Ub, ubiquitin; IP, immunoprecipitation; IB, immunoblotting; nt, nucleotides.

and 411 are the target sites of TRAF6-mediated ubiquitination.

To distinguish between these two possibilities, we examined the interaction of TRAF6 with the deletion mutants of IRF-5 as well as with the K410/K411R mutant. To this effect, cell lysates from cells transfected with MyD88, TRAF-6, IRF-5 or its mutants were immunoprecipitated with TRAF6 antibodies, and the presence of IRF-5 in precipitates was detected by immunoblotting with IRF-5 antibodies (Fig. 2D). Only the full-length IRF-5 but not any of the mutants tested interacted with TRAF6, indicating that TRAF6 interacts exclusively with the carboxyl-terminal part of the IRF-5 peptide (Fig. 2D, left panel). The data further show that the K410/K411 mutation did not affect binding of TRAF6 to IRF-5; these data indicate therefore, that K410 and K411 are direct targets of TRAF6-mediated ubiquitination. We have previously shown that the transcriptional activity of the MyD88-activated IRF-5-BMv was significantly impaired (23). This mutant has an internal deletion of 288 nucleotides representing part of exon 4, exon 5, and part of exon 6 (Fig. 2C). We have therefore tested whether IRF-5-BMv is ubiquitinated and found that this variant was compromised in its ability to be targeted for ubiquitination (Fig. 2C, lane 3) and is also compromised in its ability to interact with TRAF6 (Fig. 2D, right panel, lane 3). These data indicate that in IRF-5-BMv, the PREKKL site is not accessible for TRAF6 recognition.

To determine whether the PREKKL recognition site alone is sufficient for TRAF6 binding to IRF-5, we constructed two IRF-5 mutants which had the C′ terminus deleted just after the PREKKL domain or after the PREKKL domain and four additional amino acids (Fig. 2E). We found that both of these mutants were compromised in their ability to bind IRF-5 and consequently to be ubiquitinated (lanes 2 and 3). These data indicate that although the K410 and K411 in the PREKKL domain are the TRAF6 ubiquitination targets of IRF-5, the binding of TRAF6 to IRF-5 requires not only the PREKKL site but also the C-terminal region of IRF-5.

To determine whether the ubiquitination of IRF-5 contributes to its transcriptional activation, we used transient transactivation assays. Cells were transfected with an IFNA4 reporter plasmid together with IRF-5, IRF-5v5, IRF-5v4, or the IRF-5 K410/K411R mutant in the presence and absence of MyD88, TRAF6, and ubiquitin expression plasmids (Fig. 2F). The results showed that neither MyD88, TRAF6, nor ubiquitin alone activated the IFNA4 gene promoter (Fig. 2F, left panel, lanes 1 to 3). Also the activation by IRF-5, IRF-5v5, IRF-5v4, or the IRF-5 K410/K411R mutant alone was insignificant (lanes 5 to 8). However, in the cells expressing MyD88, TRAF6, and ubiquitin, both IRF-5 and IRF-5v5 enhanced the activity of IFNA4 promoter by about five- to sixfold (compare lane 4 and lanes 9 and 10), while the enhancement by IRF-5v4 was only about threefold (lane 11). In contrast, under the same conditions, the IRF-5 K410/K411R mutant failed to stimulate the IFNA4 promoter above the base level (lane 12), suggesting that the K410 and K411 are the primary lysine residues targeted by TRAF6 and that the ubiquitination of IRF-5 enhances its transcriptional activity. IRF-5 is activated by the TLR7 pathway (25). We therefore examined whether the activation of ectopic TLR7 by its ligand R848 would enhance the stimulation of the IFNA4 promoter by IRF-5; however, the TLR7-activated pathway did not further enhance the MyD88 activation of IRF-5 (Fig. 2F, right).

The critical role of MyD88-TRAF6-mediated ubiquitination of IRF-5 in the activation of type I IFN and proinflammatory cytokine gene transcription was also evaluated by performing reporter assays in TRAF6$^{-/-}$ MEFs (Fig. 2G). Reporter plasmids containing the IFNA4 promoter were cotransfected with IRF-5 and a combination of ubiquitin and TRAF6 plasmids into TRAF6$^{-/-}$ or wild-type MEFs (Fig. 2G). The data confirm that in the absence of TRAF6, IRF-5 expressed with ubiquitin or together with MyD88 was unable to substantially activate IFNA4 reporter plasmid in TRAF6$^{-/-}$ cells (Fig. 2G, lanes 2 and 3). However, coexpression of TRAF6 activated the reporter activity to the levels seen in wild-type MEFs (Fig. 2G, lanes 4 and 5). No activation of the IFNA4 promoter was seen in IRF-5 untransfected control cells (lanes 6 and 7). These results strongly suggested that TRAF6-mediated IRF-5 ubiquitination enhances IRF-5-mediated activation of type I IFN genes.

Association of IRF-5 with IRAK1. IRAK1 associates with MyD88 and IRF-7 and is thought to be a part of the MyD88, TRAF6, and IRF-7 complex. IRF-5 was previously shown to interact with both MyD88 and TRAF6 (28). Whether IRAK1 also associates with the IRF-5 complex is unknown. To determine whether IRF-5 associates with IRAK1, IRF-5 and IRAK1 were transfected into HEK-1A1 cells, a 293T cell line that lacks functional IRAK1 (19), with the combination of MyD88-, TRAF6-, and ubiquitin-expressing plasmids as shown in Fig. 3A. IRAK1 was immunoprecipitated from the transfected cells, and the coprecipitated IRF-5 was detected by immunoblotting. Interaction of IRAK1 and IRF-5 was detected in cells coexpressing IRF-5 and IRAK1 either alone or in the presence of TRAF6 and ubiquitin (Fig. 3A, lanes 2 and 5). However, no association between IRF-5 and IRAK1 was detected in cells expressing MyD88, indicating that MyD88 expression may disrupt the IRF-5 and IRAK1 association. This may be attributed to a competition between IRF-5 and overexpressed ectopic IRAK1 for binding to MyD88. Also HEK-1A1 cells, which lack functional IRAK1 (19), may contain additional alterations in the TLR pathway that affect formation of IRF-5, MyD88, and IRAK1 complex. Western blotting of ectopic IRAK1 indicates that IRAK1 is posttranslationally modified in the cells expressing MyD88, TRAF6, and ubiquitin. Additional studies will have to determine the nature of this modification.

We next examined whether the ubiquitination of IRF-5 was dependent on IRAK1. To this effect HEK-1A1 cells were transfected with IRF-5 and ubiquitin in combination with MyD88 and TRAF6 with or without IRAK1, as shown in Fig. 3B. We have previously shown that IRAK1 is required for the activation of IRF-5 in response to TLR7 signaling (25). His-tagged IRF-5 was affinity purified and analyzed for its ubiquitination status by SDS-PAGE. Consistent with our previous results, these results showed that IRF-5 ubiquitination is critically dependent on IRAK1. In the absence of IRAK1, MyD88-activated IRF-5 was not ubiquitinated by TRAF6 (Fig. 3B). However, when cells were reconstituted with ectopic IRAK1 together with TRAF6, robust ubiquitination of IRF-5 was detected (Fig. 3B, lane 7). A kinase-inactive mutant of IRAK1, (IRAK1 D340N) was unable to rescue the ubiquitina-

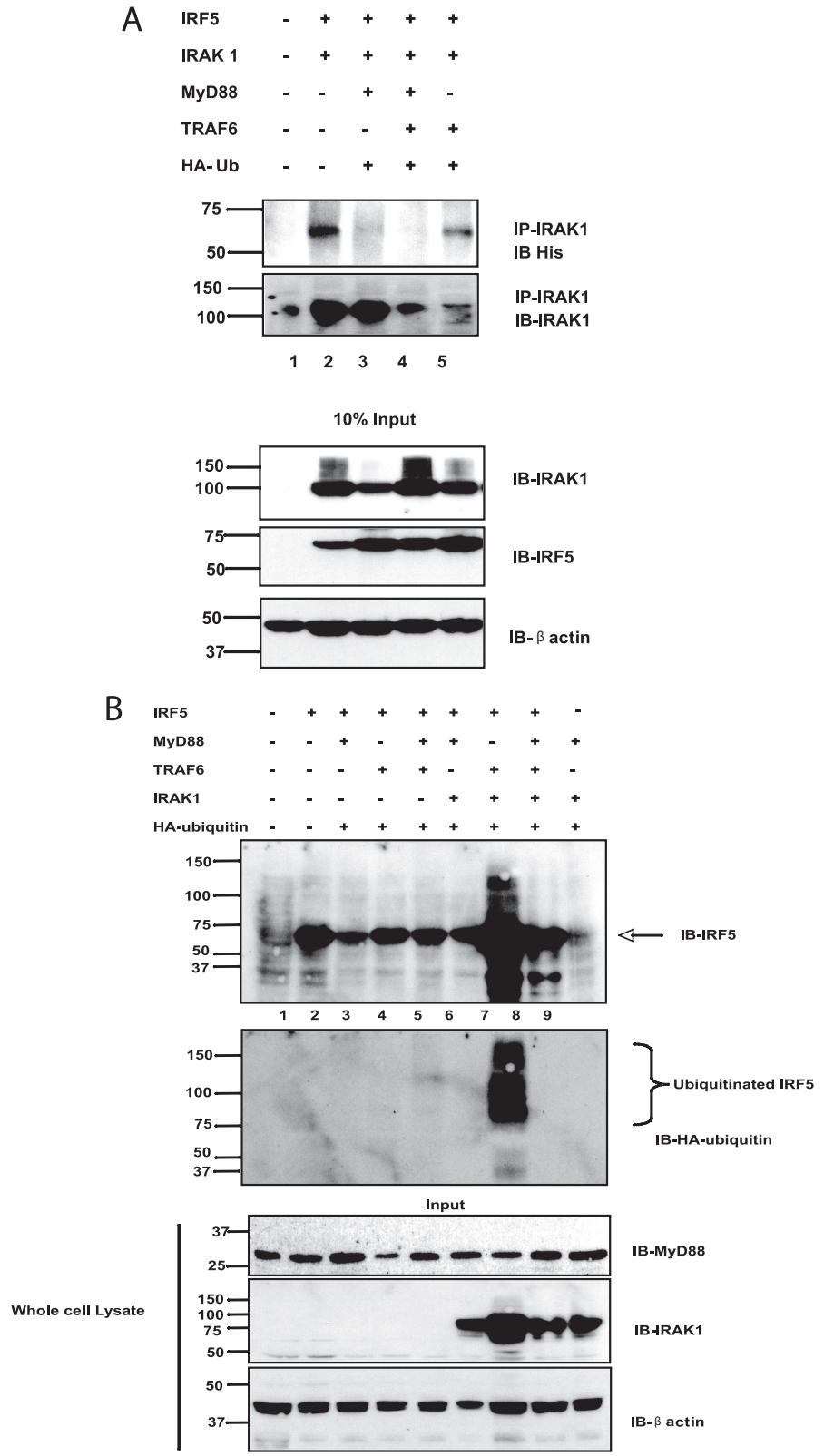
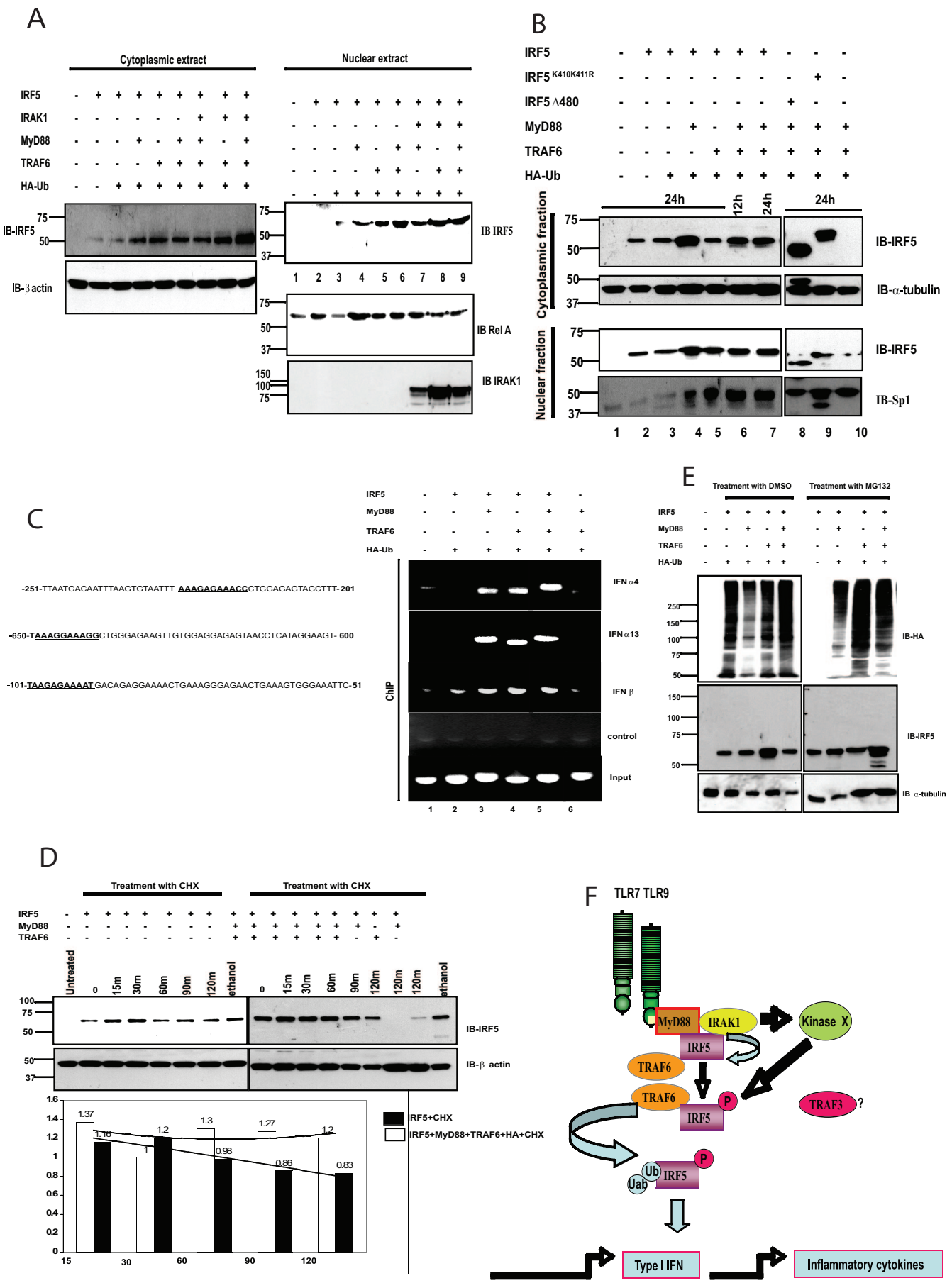


FIG. 3. IRF-5 associates with IRAK1. (A) IRAK1$^{-/-}$ 293TI1A cells were transfected with IRAK1, His-tagged IRF-5, MyD88, TRAF6, and ubiquitin (Ub). Cell lysates were immunoprecipitated (IP) with anti-IRAK1 antibody and immunoblotted with either anti-His antibody or IRAK1 as indicated. The expression of transfected plasmids in cell lysates was detected by immunoblotting with respective antibodies (lower panel). (B) 293TI1A cells, transfected with His-tagged IRF-5 (lanes 2 to 8), MyD88 (lanes 3, 5, 6, 8, and 9), TRAF6 (lanes 4, 7, and 8), or IRAK1 (lanes 6 to 9) together with HA-ubiquitin (lanes 3 to 8). IRF-5 was His purified and analyzed on an SDS gel by immunoblotting (IB) with anti-IRF-5 antibody (upper panel) and subsequently with anti-HA antibodies (middle panel). The relative levels of MyD88 IRAK1 and β-actin in input cell lysates were detected by immunoblotting with the respective antibodies.


A
Cytoplasmic extract
Nuclear extract
IRF5
IRAK1
MyD88
TRAF6
HA-Ub
IB-IRF5
IB-β actin
IB IRF5
IB Rel A
IB IRAK1
B
IRF5
IRF5 K410K411R
IRF5 Δ480
MyD88
TRAF6
HA-Ub
24h
12h
24h
24h
Cytoplasmic fraction
Nuclear fraction
IB-IRF5
IB-α-tubulin
IB-IRF5
IB-Sp1
C
-251-TTAATGACAATTTAAGTGTAATTT AAAGAGAAACCCTGGAGAGTAGCTTT-201
-650-TAAAGGAAAGGCTGGGAGAAGTTGTGGAGGAGAGTAACCTCATAGGAAGT- 600
-101-TAAGAGAAAATGACAGAGGAAAACTGAAAGGGAGAACTGAAAGTGGGAAATTC-51
IRF5
MyD88
TRAF6
HA-Ub
ChIP
IFN α4
IFN α13
IFN β
control
Input
E
Treatment with DMSO
Treatment with MG132
IRF5
MyD88
TRAF6
HA-Ub
IB-HA
IB-IRF5
IB α-tubulin
D
Treatment with CHX
Treatment with CHX
IRF5
MyD88
TRAF6
Untreated
0
15m
30m
60m
90m
120m
ethanol
IB-IRF5
IB-β actin
IRF5+CHX
IRF5+MyD88+TRAF6+HA+CHX
F
TLR7 TLR9
MyD88
IRAK1
Kinase X
IRF5
TRAF6
TRAF6
P
TRAF3
?
IRF5
P
Ub
Uab
IRF5
Type I IFN
Inflammatory cytokines

tion of IRF-5 in IRAK1-deficient cells (data not shown). Coexpression of IRF-5 with either MyD88-IRAK1 or MyD88-IRAK1-TRAF6 failed to restore IRF-5 ubiquitination (Fig. 3B, lanes 6, 8, and 9). These results are in agreement with our observations (Fig. 3A) that MyD88 may disrupt IRAK1 and IRF-5 association.

The kinase activity of IRAK1 was required for the ubiquitination of IRF-5 since the kinase-inactive mutant of IRAK1 was unable to rescue IRF-5 ubiquitination in IRAK null cells (data not shown). The requirement of IRAK1 for the IRF-5 ubiquitination could indicate that phosphorylation of IRF-5 may be a prerequisite for efficient ubiquitination. It was shown recently that IRAK1 phosphorylates IRF-7 in vitro, suggesting that IRAK1 may serve as an IRF-7 kinase; however, direct phosphorylation of IRF-7 by IRAK1 in vivo was not demonstrated. Whether IRF-5 is directly phosphorylated by IRAK1 or another IRAK1-activated kinase is not known, and experiments are under way to address this issue.

IRF-5 ubiquitination promotes nuclear localization, recruitment to the virus-responsive element (VRE) of type I IFN promoters, and stability. It was shown that IRAK1 deficiency impaired nuclear localization of IRF-7 in the MyD88 signaling pathway (29). Since our data show that ubiquitination of IRF-5 is dependent on IRAK1, we examined whether IRF-5 translocates to the nucleus in HEK-1A1 cells upon activation by MyD88. HEK-1A1 cells were cotransfected with IRF-5 and ubiquitin and combinations of MyD88- and TRAF6-expressing plasmids. Cytoplasmic and nuclear extracts were prepared at 24 h posttransfection, and the relative levels of IRF-5 were detected by immunoblotting. The data show that MyD88-activated IRF-5 was not translocated to the nucleus in HEK-1A1 cells unless the cells were reconstituted with ectopic IRAK1. The data also revealed that IRF-5 was able to translocate into the nucleus of IRAK1 null cells upon overexpression of TRAF6 (Fig. 4A) since TRAF6 works downstream of IRAK1.

To demonstrate directly that the ubiquitination of IRF-5 is necessary for or facilitates nuclear localization of IRF-5, we have followed the accumulation of IRF-5 ubiquitin-deficient mutants in the nucleus (Fig. 4B). 293T cells were transfected either with full-length IRF-5, IRF-5 ubiquitin deficient point mutants (K410/K411R), or a C-terminal deletion mutant (IRF-5Δ480, IRF-5 with a deletion of residues 480 to 539) together with a combination of MyD88, TRAF6, and ubiquitin as shown in Fig. 4B. Cells were lysed, and nuclear and cytoplasmic fractions were prepared at 12 h and 24 h posttransfections. As we have shown previously (6), low levels of ectopic IRF-5 were present in the nucleus even in unstimulated cells; however, the relative levels of nuclear IRF-5 were significantly increased in cells expressing MyD88 and TRAF6 (Fig. 4B, nuclear fraction). In contrast, in cells expressing MyD88 and TRAF6, the ubiquitin-deficient mutants IRF-5 K410/K411R and IRF-5Δ 480 accumulated in the cytoplasm (Fig.4B, lanes 8 and 9), and their relative levels in the nucleus correspond to those in unstimulated cells (compare lanes 8 and 9 and lanes 2 and 3). The relative levels of α-tubulin and Sp-1 were monitored as loading controls for the cytoplasmic and nuclear proteins, respectively. While the levels of tubulin were comparable in all the samples, the relative levels of Sp-1 were significantly higher in cells expressing MyD88 and TRAF6, suggesting that the MyD88 signaling pathway stimulates Sp-1 expression. Altogether these results demonstrate the importance of IRF-5 ubiquitination in its translocation to the nucleus.

We also examined the recruitment of IRF-5 to the VRE region of IFNA promoters and to the positive regulatory domain III/I region of the IFNB promoter (Fig. 4C) upon its activation by the MyD88-TRAF6-ubiquitin pathway. A ChIP assay was performed using a previously tested IRF-5-specific antibody. IRF-5 binding to the IFNA4, IFNA13, and IFNB promoters dramatically increased upon IRF-5 activation by MyD88 and by overexpression of TRAF6 and ubiquitin (Fig. 4C, lane 3, 4, and 5). No IRF-5 binding to the IFNA VRE was seen in controls, and only trace amounts of IRF-5 bound to the IFNB positive regulatory domain elements (Fig. 4C, lane 1, 2, and 6). These data indicate that the MyD88-mediated pathway stimulates binding of IRF-5 to the type I IFN promoters and that overexpression of TRAF6 and ubiquitin is sufficient to mimic MyD88 activation of IRF-5 and IRF-5 DNA binding activity.

It was shown that K63-linked ubiquitination increased the stability of p53 (8). We therefore wanted to determine if the K63-ubiquitinated IRF-5 was also more stable. 293T cells were transfected with IRF-5 alone or in combination with MyD88, TRAF6, and ubiquitin. Twelve hours after the transfection cells, were treated with cycloheximide (CHX) to stop de novo protein synthesis, and the relative levels of

FIG. 4. Ubiquitination of IRF-5 promotes nuclear transport, binding to type I IFN promoters and IRF-5 stabilization. (A). IRF-5 nuclear localization of IRF-5 in IRAK1$^{-/-}$ cells. 293TI1A cells were cotransfected with IRF-5, MyD88, TRAF6, and ubiquitin (Ub). Nuclear and cytoplasmic fractions were prepared 24 h after the transfections, and proteins were separated on SDS gels and immunoblotted (IB) with IRF-5 antibodies. To demonstrate equal loading, blots were stripped and immunoblotted with β-actin (cytoplasmic fraction), and the purity of the nuclear fraction was demonstrated by blotting with anti-RelA antibody. Immune blotting with anti-IRAK1 antibody was used to determine the levels of ectopic IRAK1. (B) 293T cells were transfected with IRF-5, the IRF-5 K410/K411R mutant, and the IRF-5Δ (with a deletion of residues 480 to 539) mutant, and cytoplasmic and nuclear proteins were resolved by SDS-PAGE and analyzed by Western blotting with anti-IRF-5, anti-Sp1 (nuclear fraction), and anti-α-tubulin (cytoplasmic fraction) antibody. (C) The association of IRF-5 with the promoters of the endogenous IFNA and IFNB genes was determine by ChIP assay in 293T cells transfected with IRF-5, MyD88, TRAF6, and ubiquitin as described in Materials and Methods. The sequences of the PCR-amplified promoter fragments containing the IRF-E binding site are shown. (D) Ubiquitination leads to stabilization of IRF-5. The left panel shows relative levels of IRF-5 in cells transiently expressing IRF-5, and the right panel shows IRF-5 levels in cells in which IRF-5 was coexpressed with MyD88, TRAF6, and ubiquitin. Ethanol control is a control sample cultured in the absence of CHX. Gels were scanned, and the ratios of IRF-5 to β-actin are shown as a function of time. (E) The relative levels of ubiquitinated proteins in cell lysates from 293T cells expressing IRF-5, MyD88, TRAF6, and ubiquitin and cultured with either DMSO (left panel) or 10 μM MG132 (right panel) for 6 h. The blots were reprobed with anti-IRF-5 (middle panel) and α-tubulin (lower panel) antibodies. (F). Proposed model of molecular mechanism leading to IRF-5 activation by a TLR7/9-activated, MyD88-dependent pathway.

IRF-5 were monitored for 2 h (Fig. 4D). The results showed that while the ectopic IRF-5 protein starts to be degraded as early as 60 min after CHX treatment, when IRF-5 was coexpressed with MyD88, TRAF6, and ubiquitin, there was no significant degradation of IRF-5 until 120 min posttreatment (right panel). The relative levels of β-actin at different time points of CHX treatment are shown as a control for equal protein loading. These data show that the MyD88-activated ubiquitinated IRF-5 is more stable. Whether the association of ubiquitinated IRF-5 with the other members of the MyD88-activated ternary complex also contributes to its stability is not known and cannot be excluded. Treatment with the proteasome inhibitor MG132 (Fig. 4E) did not increase significantly the levels of IRF-5 ubiquitination, compared to the untreated controls, confirming that K63-linked IRF-5 ubiquitination was not targeted for degradation by the proteasome.

DISCUSSION

We have shown in this study that IRF-5 is targeted for K63-linked polyubiquitination in an IRAK1- and TRAF6-dependent manner and that lysines 410 and 411 in the carboxyl-terminal region of IRF-5 polypeptide are the target residues for TRAF6-mediated ubiquitination. We show that TRAF6 is required for IRF-5 ubiquitination and that this activity depends on the presence of kinase-competent IRAK1. Ubiquitination of IRF-5 facilitates its transport to the nucleus and binding and activation of the promoters of the IFNA and IFNB genes (Fig. 4F). Mutations of lysines 410 and K411 to arginine localized in a putative TRAF6 consensus binding site, PXEX X(Ar/Ac), prevented ubiquitination and nuclear localization of IRF-5 K410/K411R in response to MyD88 activation while these changes did not affect binding of TRAF6. These findings suggest that the K63-linked ubiquitination of IRF-5 is a critical requirement that regulates the activity of IRF-5 in the MyD88-activated antiviral pathway. However, the ubiquitination of IRF-5 occurs at low levels, and even small degrees of IRF-5 ubiquitination can enhance IRF-5 signaling. We also show that the spliced variant IRF-5-BMv that contains a large internal deletion of 288 nucleotides in the region representing exon 5 and parts of exons 4 and 6 is not ubiquitinated by the MyD88-activated signaling pathway. TRAF6 also failed to bind the IRF-5-BMv variant, thus indicating that the internal deletion in the IRF-5 polypeptide affects its secondary structure and masks the TRAF6 recognition site. We have shown previously that the transcriptional activity of IRF-5-BMv is significantly impaired (23). In contrast, the MyD88-activated HuIRF-5v4 that contains only 48 nucleotide deletions in exon 6 is still effectively ubiquitinated and is transcriptionally active. Similar to IRF-5, IRF-7 also interacts with TRAF6 and is K63 polyubiquitinated (18) by the MyD88 signaling pathway. However, the analysis of IRF-7 ubiquitination has shown that the region between amino acids 238 and 285 of IRF-7 is important for both TRAF6 binding and polyubiquitination of IRF-7 (18). Thus, it is not clear whether the impairment of IRF-7 ubiquitination reflects the inability of TRAF6 to bind IRF-7. Also, the functional consequences of IRF-7 ubiquitination have not yet been established.

Our study provides key new insights into the molecular mechanisms of IRF-5 activation. We along with others have shown previously that IRF-5 is activated by TLR7 and TLR9 signaling, which relies on the adapter molecule MyD88 (28), but not by the TLR3 or TLR4 MyD88-independent pathway (25). TLR7 and TLR9 use only MyD88 as an adapter and activate both IRF-7 and IRF-5. MyD88 is also activated by TLR2 and TLR4, but these TLRs induce either very little IFN-β (TLR4) or none at all (TLR2) although they effectively activate NF-κB. Whether the presence of any of the additional adaptors associated with TLR2 or TLR4 (MyD88 and TIRAP with TLR2 and MyD88, TIRAP, TRIF, and Mal with TLR4) interferes with the formation of MyD88, IRAK1, TRAF6, and IRF-5 signaling complex is not known.

TRAFs are major signal transducers of the tumor necrosis factor receptor family. TRAF6 is a ubiquitin ligase-containing RING domain that was shown to be a key factor for the NF-κB activation by interleukin-1receptor, TLR, and nucleotide oligomerization domain 2 signaling pathways (2, 1). Major biological effects of TRAF signaling are mediated by activation of NF-κB and Ap-1 family members. The unique biological functions of TRAF6 are determined by its C-terminal domain that does not interact with peptides recognized by TRAF1, -2, -3, or -5. Thus, TRAF6 binds to distinct peptides from those binding TRAF2 or TRAF3 (32). TRAF6 signaling downstream from TLR7 or TLR9 involves association of TRAF6 with IRAK4 and IRAK1. Full-length IRAK1 contains three potential TRAF6 binding sites, suggesting that these TRAF6 binding sites in IRAK1 contribute to TRAF6 activation. Peptides derived from this TRAF6-interacting motif inhibited TRAF6-mediated signal transduction (32). We have shown that IRAK1, but not an IRAK1 kinase-inactive mutant, regulates TRAF6-mediated ubiquitination of IRF-5, suggesting that the function of IRAK1 is required for the activation of TRAF-6 and consequent TRAF6-mediated ubiquitination of IRF-5.

It was shown that TRAF6 also binds to TRIF (24), where it recruits TRIF-associated TBK-1 and IκB kinase ε (IKKε). However, the activation of IRF-5 by the TRIF-dependent pathway has not been observed. It was shown that phosphorylation of IRF-7 in the MyD88-mediated pathway does not require TBK-1 and is instead dependent on IRAK1. However, direct phosphorylation of IRF-7 by IRAK1 was not examined (18). IKKα was also shown to be involved in the TLR7 and TLR9 mediated induction of IFN-α. It was shown that IKKα activates IFNA promoter in synergy with IRF-7, while the induction of IFNB was not greatly modulated. It remains to be clarified whether IKKα cooperates with IRAK-1 (16) in this pathway or in the activation of IRF-5. Thus, the protein kinase that serves as an IRF-5 kinase in the MyD88-dependent pathway and the role of phosphorylation have yet to be clearly elucidated. Further studies are also required to determine whether the MyD88 and Newcastle disease virus activation of IRF-5 targets the same or distinct serine residues.

Recently another member of the TRAF family, TRAF3, has been implicated as a part of MyD88- and TRIF-dependent signaling complexes (14). A comparison of the function of TRAF3 and TRAF6 has shown that TRAF6 has a key role in MyD88 signaling but not in TRIF signaling. TLR9-induced activation of inflammatory genes was dependent on

TRAF6, while the TLR3 activation was TRAF6 independent. These authors concluded that MyD88 activation recruits both TRAF3 and TRAF6, but the TRAF6-dependent pathway results in the activation of NF-κB and participates in the induction of inflammatory cytokines while the TRAF3 pathway recruits TBK-1 and has an important role in the TRIF-dependent activation of IFN genes. Our data clearly identify IRF-5 as an additional effector of the TRAF6 pathway. Thus, in the TLR7/9 and MyD88 pathways, TRAF6 leads to activation of the IKK, mitogen-activated protein kinase, and IRF-5 pathways. However, one difference between the MyD88-mediated activation of IRF-5 and type I IFN production and NF-κB-mediated activation of inflammatory cytokines is the distinct requirement for IRAK1 in the antiviral pathway. As we have shown in this study, IRAK1 associates with IRF-5 and is required for MyD88-dependent IRF-5 ubiquitination and activation. Similarly, IRAK1 was required for activation of IRF-7 (29). However, MyD88 activation of mitogen-activated protein kinase was not impaired by IRAK1 deficiency (29), and the activity of IRAK1 was not required for NF-κB activation (19). These findings imply that the specificity of TRAF6 recruitment to the antiviral pathway may be determined by the formation of the MyD88, IRAK1, and TRAF6 signaling complex and its association with IRF-5 or IRF-7.

In summary, our study highlights the importance of IRF-5 ubiquitination in the MyD88-mediated antiviral signaling pathway. Further understanding of the molecular mechanisms involved in the IRF-5-induced inflammatory pathway will help to elucidate the role that IRF-5 plays in the pathophysiology of autoimmune diseases such as SLE.

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