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Interferon Regulatory Factor-3-mediated Activation of the Interferon-sensitive Response Element by Toll-like receptor (TLR) 4 but Not TLR3 Requires the p65 Subunit of NF-κB*

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Interferon regulatory factor (IRF) 3 is a transcription factor that binds the interferon-sensitive response element (ISRE) and is activated by Toll-like receptor 3 (TLR3) and TLR4. We have found that a dominant negative form of IκB kinase 2 and a mutant form of IκB, which acts as a super-repressor of NF-κB, blocked activation of the ISRE by the TLR4 ligand lipopolysaccharide but not the TLR3 ligand poly(I-C). TLR4 failed to activate the ISRE in mouse embryonic fibroblasts bearing a targeted deletion of p65, whereas the response to TLR3 in these cells was normal. The p65 subunit of NF-κB was detected in the lipopolysaccharide-activated but not poly(I-C)-activated ISRE-binding complex. Finally, p65 promoted transactivation of gene expression by IRF-3. These results therefore indicate that IRF-3-mediated activation of the ISRE by TLR4 but not TLR3 requires the p65 subunit of NF-κB.

The discovery of human Toll-like receptors (TLRs)[1] has increased our understanding of the molecular basis to innate immunity. TLRs allow the host to differentiate between groups of pathogens and to tailor its initial response accordingly. At least 10 different TLRs occur in humans (1). Most of their ligands have been assigned, the majority of which are so-called pathogen-associated molecular patterns. Two of the best studied TLRs are TLR4, which recognizes lipopolysaccharide (LPS) from Gram-negative bacteria, and TLR3, which recognizes the viral double-stranded RNA mimic poly(I-C) (2–5). TLRs are defined by external leucine-rich repeats and a cytoplasmic Toll/interleukin-1 receptor (TIR) domain (1). Upon stimulation the TLRs recruit TIR domain-containing adaptor molecules via homotypic interactions with receptor TIR domains. This initiates signal transduction culminating in the activation of transcription factors and an increase in immune and inflammatory gene expression. A key question concerns specificity in signal transduction by different TLRs, because although common gene sets are induced by TLRs, specific patterns of gene expression have been demonstrated for TLR2, TLR3, and TLR4, which may provide a molecular basis for the tailoring of innate immune response (6–8).

Myeloid differentiation factor 88 (MyD88) was the first TIR domain-containing adaptor to be described. It is a general adaptor for TLRs, and loss of MyD88 prevents signal transduction through most of the TLRs. The only known exceptions are TLR3 and TLR4, which both initiate a so-called "MyD88-independent" pathway (5, 9). This alternative pathway is mediated by another adaptor termed TIR domain-containing adaptor-inducing IFNβ (TRIF) or TIR-containing adaptor molecule 1 (10–13). It is involved in the activation of the transcription factor interferon regulatory factor 3 (IRF-3), which binds the interferon-sensitive response element (ISRE) and induces a subset of genes, including IFNβ (2, 14, 15). Although TRIF is recruited to both TLR4 and TLR3, the recently described TRIF-related adaptor molecule (16), also called TIR-containing adaptor molecule 2 (17), which activates IRF-3 as well as NF-κB, is specific for TLR4 signaling and presumably acts upstream of TRIF. TLR4 also recruits MyD88 and an additional adaptor named MyD88 adapter-like (Mal) or TIR domain-containing adapter protein, which is also required for TLR2 signaling (18–21). Both Mal and MyD88 would appear to be involved in the rapid activation of NF-κB by TLR4, whereas TRIF, in addition to regulating IRF-3, mediates later activation (12, 13, 20, 21).

In its inactive form IRF-3 is constitutively present in a latent cytoplasmic pool. Upon stimulation with poly(I-C), IRF-3 becomes phosphorylated in its C terminus, which presumably reveals the previously hidden dimerization domain (22). LPS does not induce C-terminal phosphorylation but appears to causes N-terminal phosphorylation (23, 24). The nature of this phosphorylation and the responsible kinase is still uncertain. In the case of the C-terminal phosphorylation two IκB kinase (IKK)-related proteins, IKKε and TANK-binding kinase 1, have recently been identified as possible components of the virus- and TLR3-activated kinase complex for IRF-3 (25, 26).

In this study we have found a key role for the NF-κB subunit p65 in the IRF-3-mediated induction of the ISRE by TLR4 but not TLR3. p65 promoted transactivation of gene expression by IRF-3. TLR4 and TLR3 therefore differ in their mechanism of ISRE induction.

EXPERIMENTAL PROCEDURES

Cell Culture, Plasmids, and Reagents—HEK293 and U373 cell lines were purchased from the Centre for Applied Microbiology & Research (Salisbury, UK), the HEK293-TLR3 cell line was kind gift of Katherine Fitzgerald (University of Massachusetts Medical School). The mouse embryonic fibroblasts (MEFs) with a targeted deletion in p65 and wild type MEFs were a kind gift from Ron Hay (University of St. Andrews, Fife, Scotland), and MEFs with a deletion of the β subunit of the interferon-α/β receptor on a 129SV/Ev genetic background and their parental MEFs were a gift from Otto Haller (University of Freiburg, Freiburg, Germany). The cells were grown in Dulbecco's modified Ea-

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[1] The abbreviations used are: TLR, Toll-like receptor; LPS, lipopolysaccharide; TIR, Toll/interleukin-1 receptor; IFNβ, interferon β; IRF, interferon regulatory factor; ISRE, interferon-sensitive response element; IKK, IκB kinase; MEF, mouse embryonic fibroblast; hTLR, human TLR; CBP, cAMP-responsive element-binding protein-binding protein.

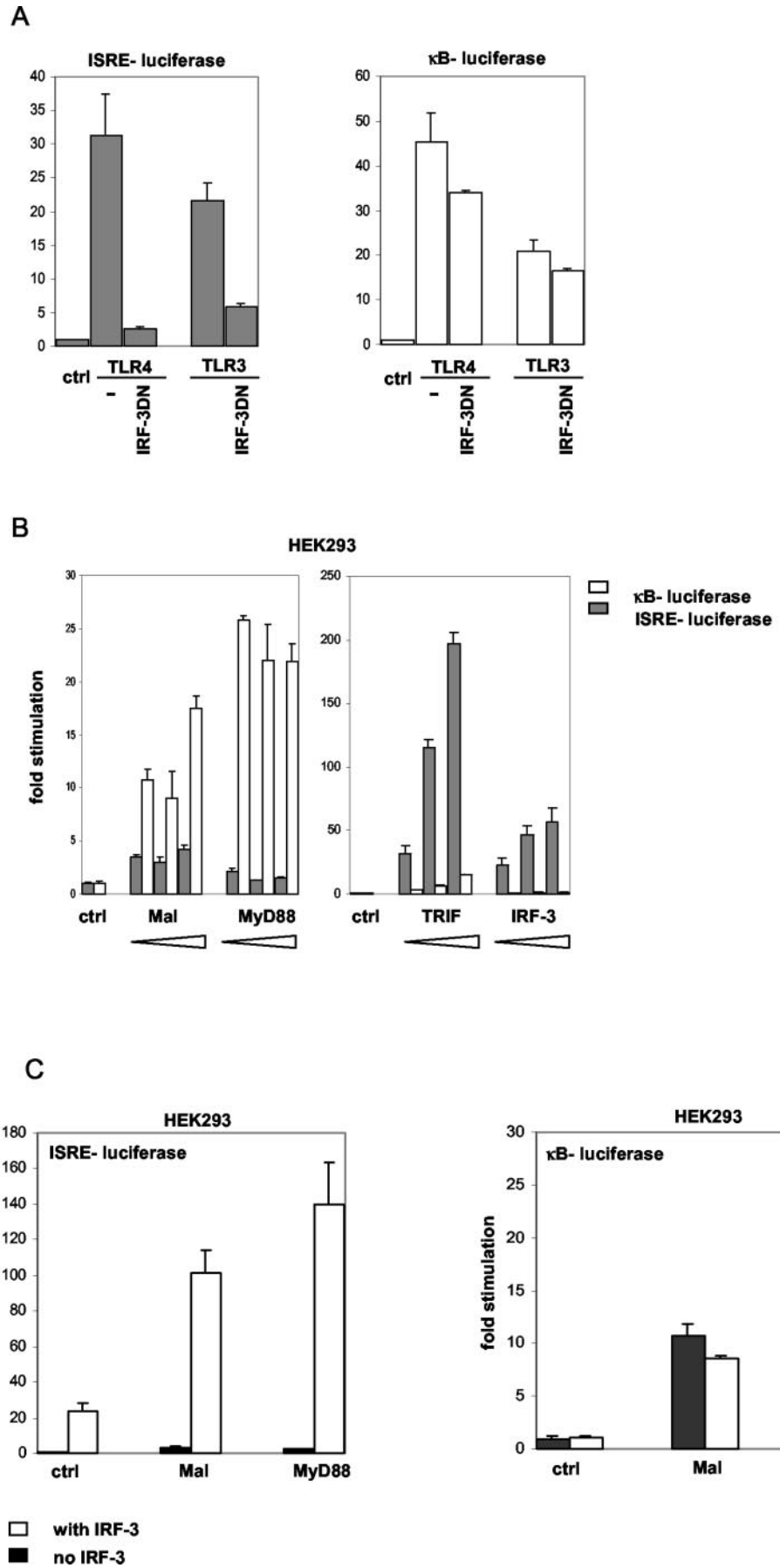

FIG. 1. **Synergism between MyD88 or Mal and IRF-3 in the activation of the ISRE.** *A*, the inhibitory effects of plasmids encoding dominant negative versions of IRF-3 (ΔNIRF-3, 50 ng) were tested in HEK293 cells that were co-transfected with 100 ng of the ISRE- or κB luciferase constructs and CD4-TLR4 (50 ng) (*left panel*) and in HEK-TLR3 cells, which were stimulated for 6 h with poly(I-C) (25 μg/ml) to induce

gle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, and 100 units ml^{-1} gentamycin and maintained at 37 °C in a humidified atmosphere of 5% CO_2, 200 mg ml^{-1} of the neomycin analog G418 was added to maintain HEK293-TLR3 cells. The cells were seeded at 0.5×10^5 to 10^5 ml^{-1} for experiments and treated as indicated in figure legends. LPS from *Escherichia coli* serotype O26:B6 and poly(I-C) were purchased from Sigma.

The NF-κB luciferase construct bearing five repeats of the κB consensus was a gift from Dr. R. Hofmeister (Universitaet Regensburg, Regensburg, Germany). The ISRE luciferase construct, which has five repeats of the ISRE sequence from the ISG15 promoter, was purchased from Clontech (Palo Alto, CA). Constructs for the IRF-3 transactivation assay, p-55UAS$_G$Luc, Gal4-DBD, and Gal4-IRF-3 were a kind gift of Takashi Fujita (Tokyo Metropolitan Institute of Medical Science, Tokyo, Japan). The chimeric CD4-TLR4 and CD4-TLR3 expressing vectors were a gift from Ruslan Medzhitov (Yale University School of Medicine). Mal was expressed from pDC304, and MyD88 was from pCDNA3.1. The plasmid encoding TRIF was a kind gift from Shizuo Akira (Osaka University, Osaka, Japan). The construct comprising hemagglutinin-tagged p65 and the IκB-SR expression vector were kind gifts from Keith Ray (GlaxoSmithKline, Stevenage, UK). Constructs encoding the dominant negative version IKK2, IKK2KA, and human TLR3 (hTLR3) were obtained from Tularik (San Francisco, CA). Expression constructs bearing IRF-3 and ΔNIRF-3 were kind gifts of John Hiscott (McGill University, Montreal, Canada).

Transfection-based Reporter Gene Assays—HEK293, U373, and MEFs were seeded at $1–2 \times 10^4$ cells $well^{-1}$ in 96-well plates, incubated overnight, and transfected using GeneJuice transfection reagent (Novagen, Madison, WI) according to the manufacturer's instructions with a total amount of 300 ng of DNA $well^{-1}$ comprising 100 ng of reporter gene construct, plasmid DNA of interest, and empty vector as filler DNA. For transactivation assays 80 ng of p-55UAS$_G$Luc were applied in combination with 100 ng of either Gal4-DBD or Gal4-IRF-3 and additional plasmids as indicated in figure legends. The cells were lysed in passive lysis buffer (Promega, Southampton, UK) for 15 min. At 24 or 48 h post-transfection, the extracts were monitored for firefly luciferase activity following standard protocols. 40 ng $well^{-1}$ *Renilla reniformis* luciferase construct was used as internal control for transfection efficiency. The activities were expressed as fold activation over unstimulated empty vector controls (*ctrl*). The experiments were carried out in triplicate with *error bars* indicating standard deviations.

Electrophoretic Mobility Shift Assays—U373 cells were grown in 15-cm dishes and treated as indicated in figure legends, and their nuclear extracts were prepared as previously described with minor modifications (27). Specifically, the samples were homogenized using a Dounce homogenizer (80 strokes at 4 °C). Following the addition of the high salt buffer, the samples were rotated for 30 min at 4 °C. 10 μg of nuclear protein was incubated for 30 min with 10,000 cpm of double-stranded [γ-^{32}P]ATP ISRE oligonucleotide (5′-GAT CGG GAA AGG GAA ACC GAA ACT GAA-3′) in hybridization buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM dithiothreitol, 4% glycerol, and 100μg ml^{-1} nuclease free bovine serum albumin) containing 2 μg ml^{-1} of poly(dI-dC) as nonspecific competitor. Supershifting antibodies specific for p65 (SC-8008X) and IRF-3 (SC-9082X) were added to the nuclear extracts 1 h prior to hybridization with the oligonucleotide. The samples were kept on ice at all stages. Protein-DNA complexes were run on a 5% native polyacrylamide gel, and complex formation was detected with autoradiography.

RESULTS

The TLR4 Adaptors MyD88 and Mal Enhance ISRE-dependent Gene Expression—We were interested in comparing IRF-3 activation by TLR4 and TLR3. To examine this process we used three systems comprising transfection of HEK293 cells with a CD4-TLR4 fusion protein, which is constitutively active, HEK293 cells rendered sensitive to poly(I-C) by stable transfection with TLR3, and addition of the ligands LPS and poly(I-C) to U373 cells, which express TLR3 and TLR4 constitutively. Fig. 1*A* demonstrates that transfection with a plasmid encoding a dominant negative version of IRF-3 blocked CD4-TLR4 and TLR3/poly(I-C)-stimulated induction of a reporter gene, luciferase, linked to five ISRE sites from the ISG15 promoter (*left-hand panel*) but did not inhibit the expression of luciferase linked to five NF-κB-binding sites (*right-hand panel*). These results indicate that the ISRE luciferase response involves IRF-3.

We next probed the link between the adaptors MyD88, Mal, and TRIF with IRF-3. Fig. 1*B* demonstrates how overexpression of each of the proteins on their own affected ISRE- or κB-linked reporter gene expression. In agreement with other studies (10, 11) expression of TRIF led to the strongest activation of the ISRE, causing a 200-fold induction over controls. By comparison, MyD88 and Mal had only marginal effects on the ISRE but were much better inducers of NF-κB when compared with TRIF. Overexpression of IRF-3 only induced the ISRE response. However, as shown in Fig. 1*C*, when concentrations of Mal or MyD88 unable to activate the ISRE were co-expressed with IRF-3, a clear synergy was observed in ISRE induction (Fig. 1*C*, *left-hand panel*). Mal enhanced the IRF-3 alone response by 5-fold, whereas MyD88 had a 7-fold effect. IRF-3 had no effect on NF-κB either alone or in combination with Mal or MyD88, as expected (Fig. 1*C*, *right-hand panel*).

NF-κB Is Required for IRF-3 Activation by LPS but Not poly(I-C)—The synergism observed between MyD88 or Mal and IRF-3 with respect to ISRE activation suggested that TLR4 signaling to IRF-3 might involve NF-κB, because Mal and MyD88 are both NF-κB activators. To examine this we tested specific inhibitors of the NF-κB pathway for their effects on TLR4-mediated and, for comparison, TLR3-mediated signal transduction to the ISRE. We used IκB-SR, a mutant form of IκBα (S32A/S36A), which cannot undergo phosphorylation and acts as a super-repressor of NF-κB, and a kinase inactive mutant of IKK 2 (IKK2KA), which acts as a dominant negative inhibitor. Interestingly, both agents inhibited CD4-TLR4-stimulated ISRE and, as expected, NF-κB-mediated gene induction (Fig. 2*A*). Strikingly, neither had an effect on ISRE activation by poly(I-C) in TLR3-transfected HEK293 cells but, as expected, blocked NF-κB activation, although the inhibitory effect of IKK2KA reached a maximum of only 50% (Fig. 2*B*). Similar results were obtained when ISRE- and κB-dependent expression was measured in U373 cells. LPS-mediated activation of the ISRE was repressed by both IKK2KA and IκB-SR, whereas the effect of poly(I-C) was not impaired by either of these agents (Fig. 2*C*, *left-hand panel*). NF-κB activation by LPS and poly(I-C) in U373 was blocked by both IKK2KA and IκB-SR (*right-hand panel*) with IKK2KA again having a lesser effect than the IκB-SR on the poly(I-C) response.

TLR4 Fails to Induce ISRE-dependent Gene Expression in the Absence of p65—We further tested the significance of NF-κB in the TLR4 response using p65-deficient cells. Fig. 3 (*left-hand panel*) demonstrates how CD4-TLR4 activates NF-κB in wild type MEFs, whereas the effect in p65-deficient MEFs was impaired, as expected. TLR3 signaling to NF-κB

expression from ISRE and κB luciferase constructs (*right panel*). *B*, HEK293 cells were co-transfected with the ISRE (*gray histobars*) or the κB luciferase constructs (*white histobars*) and increasing amounts (25, 50, and 100 ng) of plasmids encoding MyD88, Mal, TRIF, and IRF-3. *C*, expression of the ISRE and the κB luciferase constructs in HEK293 cells was assayed when plasmids encoding MyD88 (25 ng) or Mal (25 ng) were expressed with (*white histobars*) or without (*black histobars*) co-transfected IRF-3 (25 ng). In all cases 0.5×10^6 cells were transiently transfected with a total of 300 ng of DNA in 96-well microtiter plates. In all experiments, the cells were transfected for up to 48 h. Unstimulated cells transfected with an empty vector was used as control (*ctrl*). The measured luciferase reporter gene activity values were normalized for transfection efficiency with *R. reniformis* luciferase. The data shown are the means ± standard deviations from triplicate determinations. All of the results shown are representative of three separate experiments.

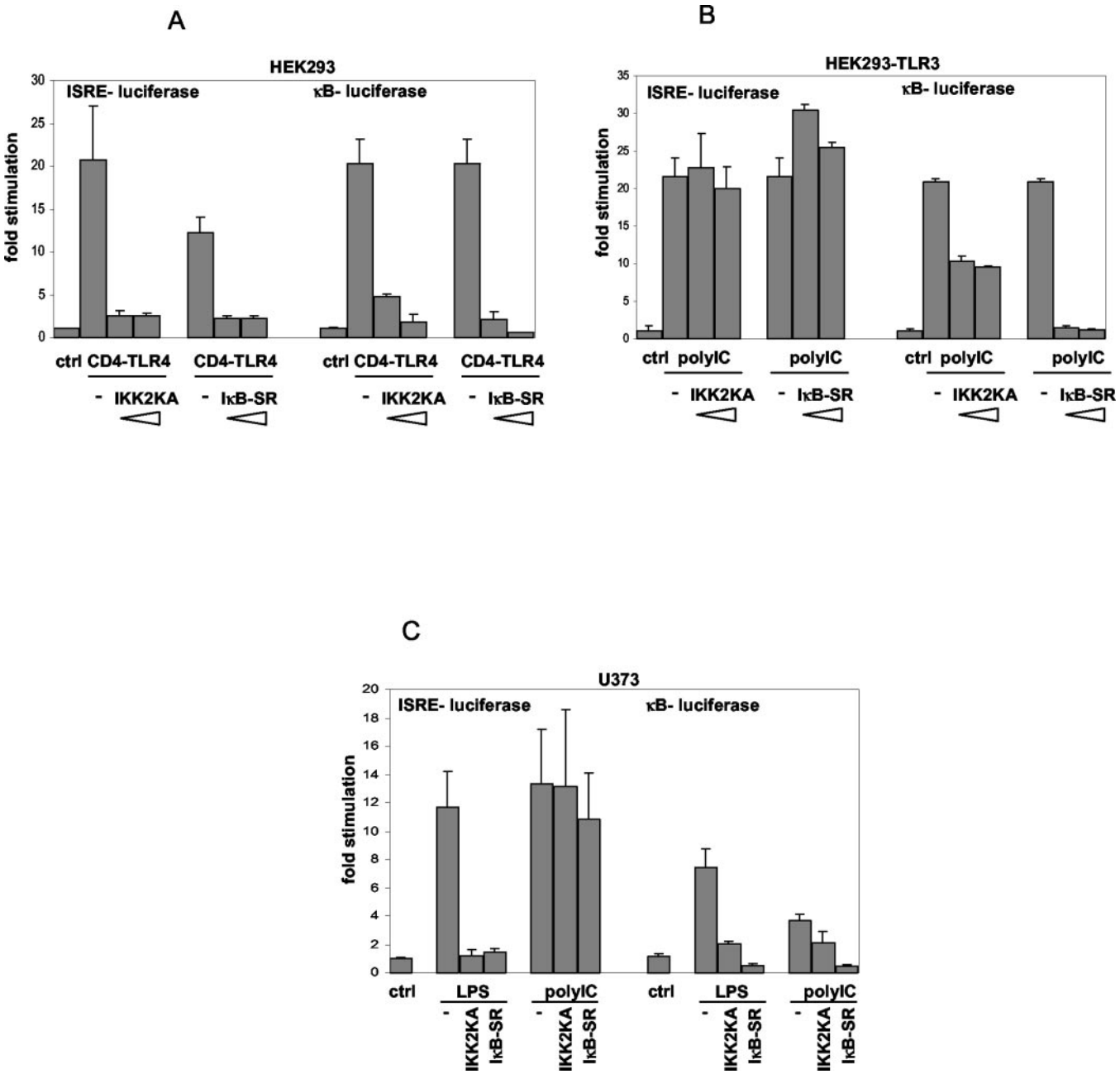

FIG. 2. **Effects of IKK2KA and the IκB-SR on ISRE activation by TLR4 and TLR3.** *A*, 0.5× 10^6 HEK293 cells were co-transfected with plasmids encoding CD4-TLR4 (100 ng), ISRE or κB luciferase constructs, and increasing amounts (10 and 25 ng) of plasmids encoding dominant negatives IKK2KA and IκB-SR. *B*, 0.5× 10^6 HEK293-TLR3 cells transfected with either the ISRE or the κB luciferase construct and increasing amounts (10 and 25 ng) of the dominant negative IKK2KA and IκB-SR were stimulated with poly(I-C) (25μg/ml) for 6 h. *C*, 10^5 U373 cells were co-transfected with either the ISRE or the κB luciferase construct and the dominant negative IKK2KA (60 ng) or IκB-SR (60 ng). The cells were stimulated with LPS (500 ng/ml) or poly(I-C) (25 μg/ml) for 6 h. After up to 48 h of transfection, the cell lysates in all experiments were measured for luciferase reporter gene activity, and the retrieved data were normalized to *R. reniformis* luciferase values. Unstimulated cells transfected with an empty vector was used as control (*ctrl*). The data shown are the means ± standard deviations from triplicate determinations and are representative of two to three experiments.

activated by poly(I-C) in MEFs transfected with TLR3 was decreased in p65-deficient cells. Importantly, a different result was obtained using the ISRE luciferase construct, as shown in Fig. 3 (*right-hand panel*). The ISRE response to TLR4 was almost abolished in the p65-deficient MEFs relative to wild type controls. We were unable to test LPS itself on MEFs because in wild type cells the activation of the ISRE was marginal (data not shown). The effect of poly(I-C), however, was similar in wild type and in p65-deficient cells. This result provides additional evidence that NF-κB, and in particular the p65 subunit, is required for signaling by TLR4 but not TLR3 to the ISRE.

p65 Interacts with IRF-3 at the ISRE and Promotes Transactivation—We next tested whether p65 was part of the IRF-3-containing ISRE activation complex. We used the ISRE from the ISG15 promoter (as used in the ISRE luciferase construct) in an electrophoretic mobility shift assay on nuclear extracts from LPS- or poly(I-C)-stimulated U373 cells. As shown in Fig. 4*A*, we observed complex formation at the ISRE oligonucleotide following stimulation with either LPS (*lane 4*) or poly(I-C) (*lane 7*). Incubation of the extract with an IRF-3-specific antibody abolished DNA binding, confirming the presence of IRF-3 in each complex (*lanes 5* and *8*). Importantly, a p65-specific antibody prevented ISRE complex formation in extracts from LPS-

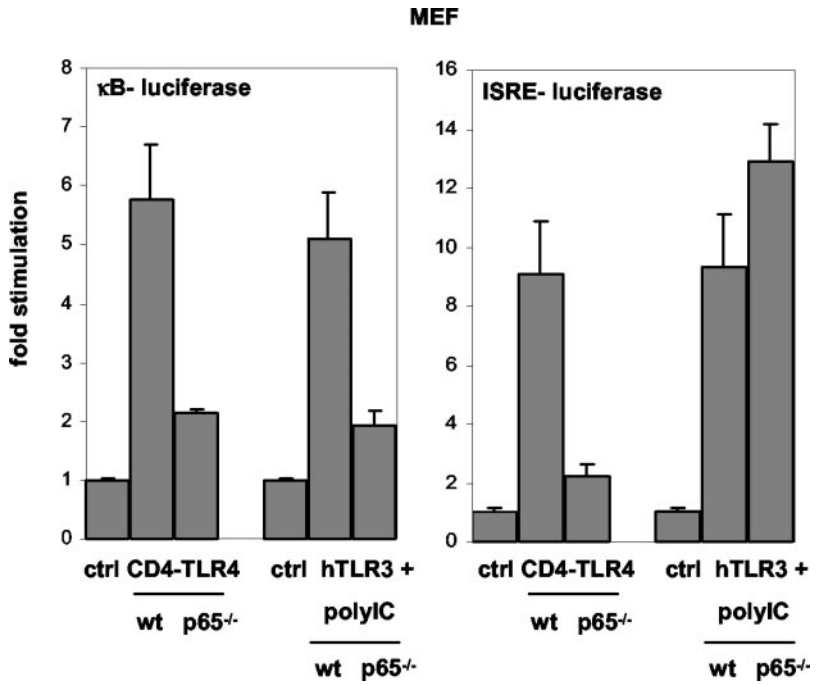

FIG. 3. **The p65 subunit of NF-κB is required for TLR4-induced activation of the ISRE.** 0.5×10^6 wild type MEFs (*wt*) or MEFs with a targeted deletion of p65 (*p65−/−*) were seeded in 96-well microtiter plates and transfected with the ISRE or κB luciferase constructs (100 ng) with either CD4-TLR4 (50 ng) or hTLR3 for 24 h. The cells transfected with hTLR3 (0.5 ng) were subsequently stimulated with 1μg/ml poly(I-C) for 7 h. Unstimulated cells transfected with an empty vector were used as control (*ctrl*). Luciferase activity was determined in cell lysates and normalized for transfection efficiency with *R. reniformis* luciferase activity. The data shown are the means ± standard deviations from triplicate determinations. An identical result was observed in a subsequent experiment.

treated cells (*lane 6*) but had only a marginal effect on extracts from poly(I-C)-treated cells (*lane 9*). The supershifting ability of the p65-specific antibody was confirmed with a κB site-containing oligonucleotide and LPS-stimulated U373 and THP-1 nuclear cell extracts (data not shown).

We also addressed what role p65 might have on the functioning of IRF-3. For this we used an IRF-3 transactivation assay. This assay involved transfecting cells with a plasmid encoding a fusion protein comprising the transactivation domain of IRF-3 fused to the DNA-binding domain of Gal4. When activated, this fusion protein drives a Gal4-controlled luciferase reporter gene co-transfected into the cells. Fig. 4*B* demonstrates that increasing expression of p65 in HEK293 cells dose-dependently promoted IRF-3-mediated transactivation. This effect was blocked by the IκB-SR but not the dominant negative IKK2KA (Fig. 4*C*). Overexpressed IκB-SR presumably sequesters p65 in the cell, thereby preventing it from enhancing IRF-3 transactivation activity. Taken together these data indicate that p65 and IRF-3 interact in a complex at the ISRE as a consequence of TLR4 but not TLR3 signaling and that p65 promotes transactivation by IRF-3.

Finally, we wanted to exclude the possibility that activation of the ISRE in our experiments is predominantly driven via a type I interferon feedback loop through activation of ISGF3. We therefore transfected MEFs bearing a targeted disruption of the β subunit of the interferon-α/β receptor ($IFNAR1^{-/-}$ MEFs) and their corresponding wild type MEFs with the κB and ISRE luciferase constructs and tested TLR3 and TLR4 responses. TLR4 and TLR3 signaling to either ISRE or NF-κB was comparable in both $IFNAR1^{-/-}$-deficient and wild type cells (Fig. 4*D*), indicating that the ISRE construct serves primarily as an IRF-3 read-out. Consistent with our data in HEK293 and U373 cells, upon co-transfection with the IκB-SR only the TLR4-stimulated ISRE response was decreased, whereas TLR3 signaling remained unaltered (Fig. 4*E*). It should be noted that the parental MEFs of the $IFNAR1^{-/-}$ MEFs, although clearly evident, were less responsive compared with the wild type MEFs, which were the corresponding controls for the p65-deficient cells.

DISCUSSION

The results from this study indicate a difference between TLR4- and TLR3-mediated activation of the ISRE. TLR4 has an absolute requirement for NF-κB in this process, with p65 occurring in the ISRE binding complex with IRF-3, whereas the TLR3-induced ISRE response is NF-κB-independent. We first suspected NF-κB involvement in the pathway to IRF-3 from TLR4 when we observed that Mal or MyD88 synergize with overexpressed IRF-3 in ISRE activation. Both of these adaptors target NF-κB. NF-κB involvement was confirmed by the inhibitory effect of IKK2KA and IκB-SR on the TLR4 response and most compellingly in cells from p65-deficient mice, which were unresponsive in terms of TLR4 signaling to the ISRE but were normal for TLR3.

The necessity for cooperation between NF-κB and IRF-3 has been outlined in other studies (14, 28, 29). Interestingly, p65 is redundant for induction of the IFNβ promoter after poly(I-C) data stimulation but is absolutely essential for LPS-stimulated IFNβ expression (30). These observations at a minimum indicate that NF-κB and IRF-3 must cooperate in TLR4 signaling but not TLR3 signaling to a gene whose promoter contains an ISRE but also support our evidence for a function for p65 in TLR4- but not in TLR3-mediated ISRE activation. Additionally, in an earlier study it has been shown that the necessity for p65 at the IFNβ promoter could be overcome with a chimera comprising the N-terminal DNA-binding domain of IRF-3 and the N-terminal p65 transactivation domain (14). This again argues for a direct cooperation between IRF-3 and p65 at the ISRE, with the role of p65 to promote transactivation. In support of this model we found that the inhibition of the NF-κB pathway in $IFNAR1^{-/-}$ MEFs cells blocks TLR4 signaling to the ISRE. Additionally, we detected both p65 and IRF-3 in the LPS/TLR4-induced activation complex at the ISRE.

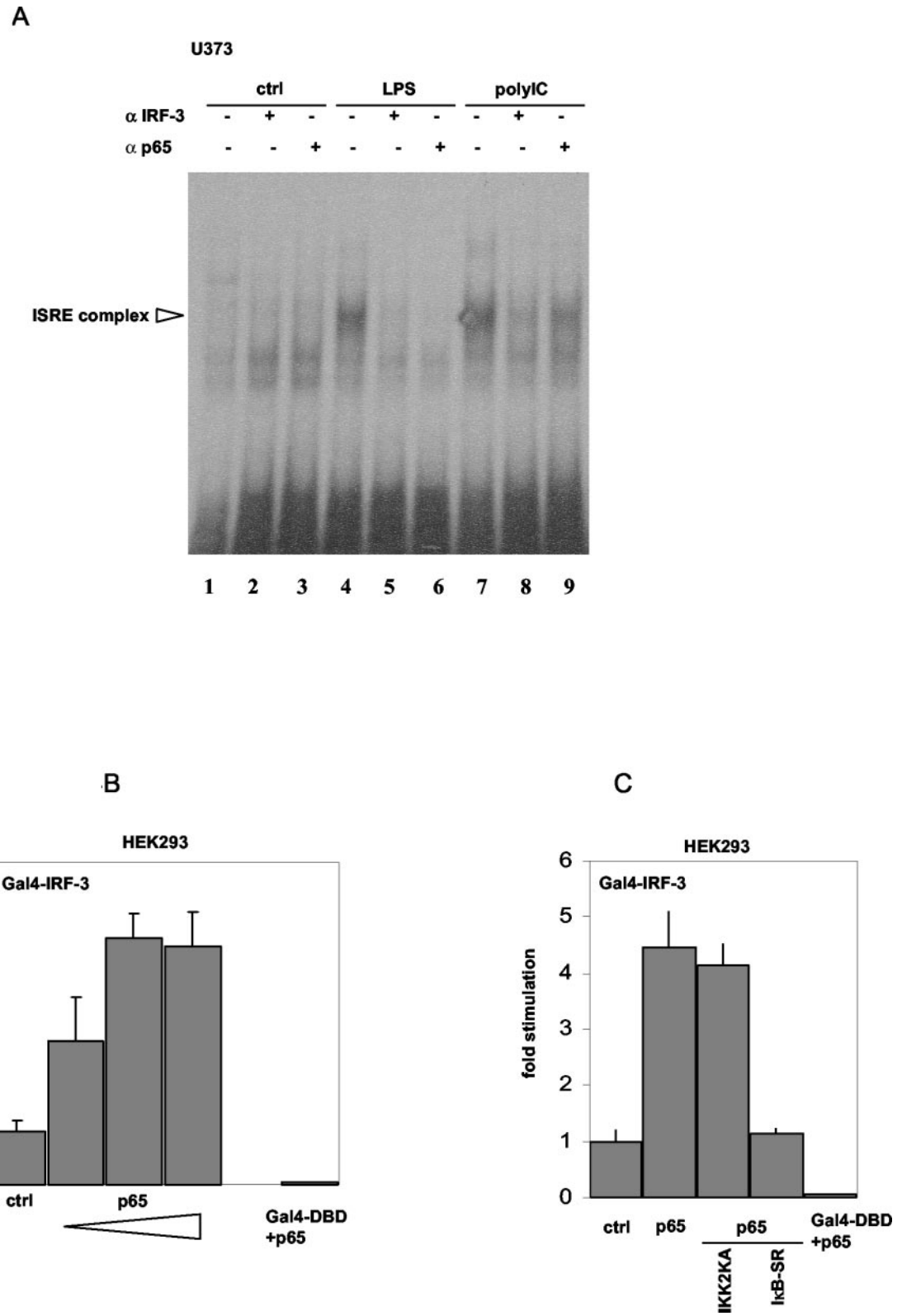

FIG. 4. **Interaction between NF-κB and IRF-3.** *A*, nuclear extracts prepared from 10^5 U373 cells treated for 6 h with LPS (1 μg/ml) or poly(I-C) (25 μg/ml) or left untreated were analyzed in a electrophoretic mobility shift assay using an oligonucleotide comprising the ISRE from the ISG15 promoter. The *arrow* indicates protein/DNA complexes. The extracts were preincubated with antibodies against IRF-3 or p65 as indicated. These results are representative of three separate experiments. *B*, 10^6 HEK293 cells were co-transfected with a construct bearing the Gal4 upstream activation sequence fused to the luciferase reporter gene (100 ng), an expression vector for Gal4-IRF-3 (80 ng), or the Gal4 DNA-binding domain on its own Gal4-DBD (80 ng) and expression vector for p65 in increasing amounts (5, 25, and 50 ng). *C*, 10^6 HEK293 cells transfected with Gal4 luciferase construct (100 ng), the expression vector for Gal4-IRF-3 (80 ng), and p65 (50 ng) were additionally transfected with expression vectors for the dominant negative form of IKK2 (IKK2KA 20 ng) and the IκB-SR (20 ng). *D*, 0.5× 10^6 wild type MEFs (*wt*) or MEFs with bearing a deletion of IFNAR1 (*IFNAR1*$^{-/-}$) were seeded in 96-well microtiter plates and transfected with 100 ng of the ISRE-or the κB luciferase constructs and with either CD4-TLR4 (60 ng) or hTLR3 (0.5 ng) for 24 h. The cells transfected with hTLR3 were stimulated with 1μg/ml poly(I-C) for 7 h. *E*, IFNAR1$^{-/-}$ MEFs were co-transfected with ISRE luciferase, CD4-TLR4, or hTLR3, respectively, and increasing doses of IκB-SR (10 ng and 30 ng). Unstimulated cells transfected with an empty vector were used as control (*ctrl*). In all experiments cell lysates were prepared 24 h after transfection

An interaction between p65 and IRF-3 at the ISRE could be mediated by the IRF-3 co-activators cAMP-responsive element-binding protein-binding protein (CBP) or p300. IRF-3 relies on recruitment of these co-activators not only to reduce export from the nucleus but moreover for transcriptional activation (15, 31). The histone acetylase function of CBP is vital for binding to the ISRE (32). CBP has been shown to interact with both IRF-3 and p65 (31, 33, 34) and to recruit the two transcription factors to distinct sites on CBP (35, 36). Simultaneous binding of p65 with IRF-3 could have a synergistic effect and possibly alter the conformation or steric orientation of IRF-3 at CBP to modify transcriptional activity of the complex. It is possible that other subunits of NF-κB, such as c-Rel or RelB, can also bind to this complex and promote ISRE activity, and we are currently investigating their involvement. However, because of the availability of p65-deficient cells, we focused on the role of p65 in this study.

The mechanism by which LPS activates IRF-3 via TLR4 is

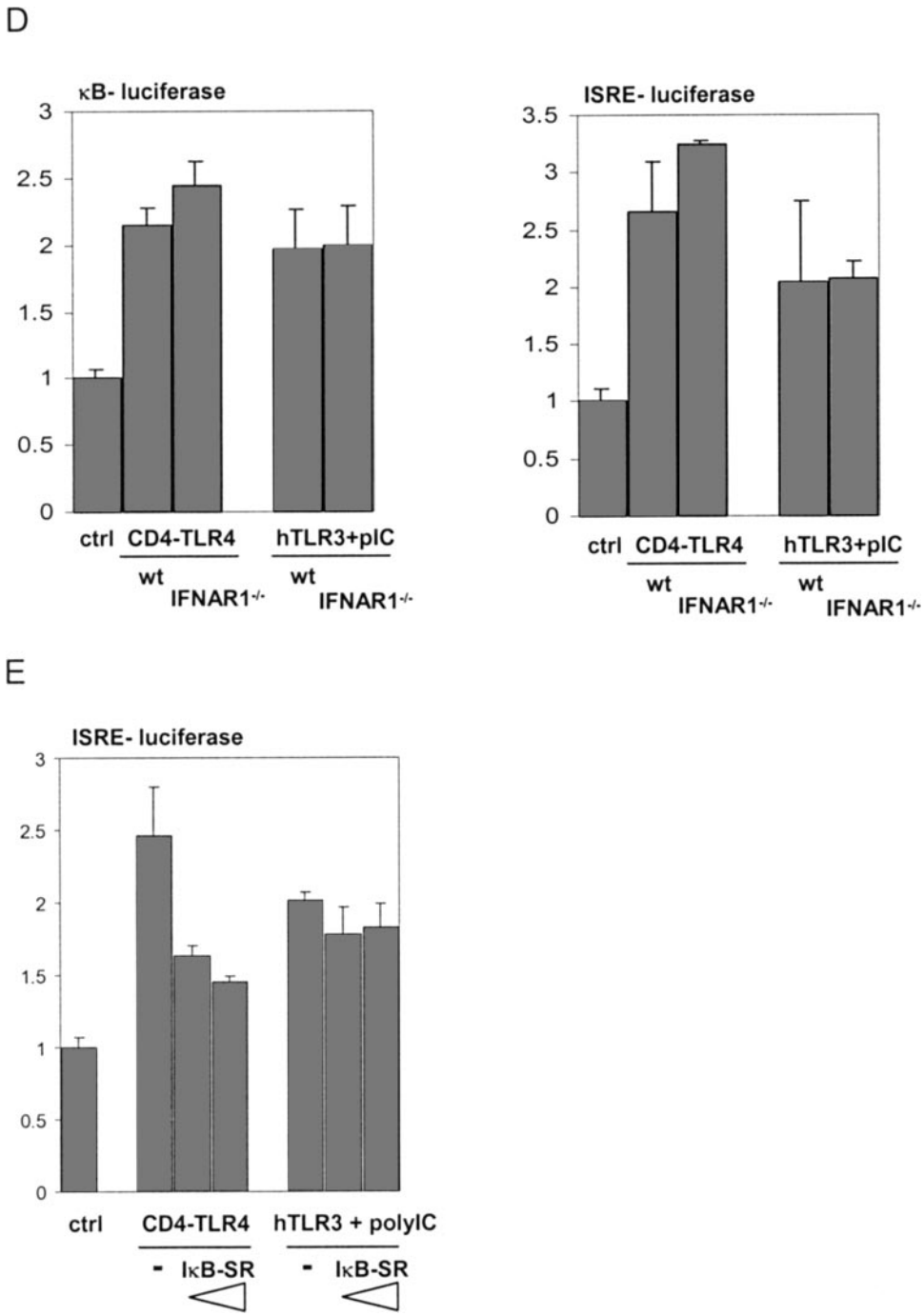

FIG. 4—*continued*

and assayed for luciferase reporter gene activity, and the obtained values were normalized against the *R. reniformis* luciferase activity for transfection efficiency. Luciferase activity in cells transfected with a Gal4-DBD expression vector was in all cases below the control values of cells transfected with the Gal4-IRF-3 construct. The data shown are the means ± standard deviations of triplicate determinations. The results shown are representative of three independent experiments.

still uncertain, although other studies have also indicated differences between LPS and viral stimuli or poly(I-C) in this process (23, 37, 38). LPS appears to cause N-terminal phosphorylation of IRF-3, whereas the activation induced by poly(I-C) is more potent and results in phosphorylation of the C terminus (23, 37). It is possible that IRF-3 phosphorylated in its C terminus does not require p65 for it to enhance transcription, whereas N-terminally phosphorylated IRF-3 does. Recently, two independent studies have implicated two members of the IKK family, IKKε and TANK-binding kinase 1, as crucial mediators in virus- or poly(I-C)-induced IRF-3 activation and have suggested that IKKε is an activating kinase for IRF-3 and IRF-7 (25, 26). This process seems to be separate from any effect these kinases might have on IKK2, which is a substrate for both IKKε and TANK-binding kinase 1. IKK2 was shown not to be capable of phosphorylating IRF-3 when immunoprecipitated from cells. Our evidence also rules out a role for IKK2 in the IRF-3 activation process triggered by poly(I-C). With respect to LPS it has been shown that ISRE binding activity induced by LPS in IKKε-deficient MEFs was unaltered compared with wild type MEFs, which argues against a role for IKKε in LPS-dependent IRF-3 activation, consistent with the evidence that LPS does not cause C-terminal phosphorylation (39). It has, however, been suggested that TRIF is involved in IKKε activation (26) and is required for IRF-3 activation by LPS, as revealed by the failure of LPS to induce IRF-3 dimerization in TRIF-deficient cells (12, 13). Further experiments will be needed to resolve this issue, and it is likely that there is additional complexity in adaptor usage in this process. Furthermore because LPS can still activate the ISRE in the absence of Mal and MyD88 (20, 21), TRIF and/or TRIF-related adaptor molecule (16), also known as TIR-containing adaptor molecule 2 (17) or TIR domain-containing adapter protein (40), may provide the signal to NF-κB in the absence of MyD88 and Mal required for ISRE activation by TLR4. The failure of TLR2 to activate IRF-3 (8) is presumably due to the fact that it signals via MyD88 and Mal and does not utilize TRIF (20, 21).

IRF-3 activation upon LPS stimulation has also been demonstrated in Gal4-IRF-3 transactivation studies and in nuclear translocation assays (41, 42). How LPS triggers these events is still not known. One possibility we are exploring is that p65 and IRF-3 interact directly, or via CBP as stated above, and is required for these responses.

Although the signaling pathways induced by TLR4 and TLR3 therefore differ, both lead to ISRE activation allowing for IFNβ expression. Clearly, the ISRE can be activated by different transcription factor complexes including IRF-7 and ISGF3, a trimeric complex comprising STAT1, STAT2, and IRF-9 (22, 43, 44). Its promiscuity may allow for fine-tuning of responses in different biological contexts. It is also possible that the p65/IRF-3 dimer targets an additional set of genes, increasing the repertoire of responses to LPS.

LPS induces IRF-3- and NF-κB-dependent production of IFNβ, which in an autocrine manner via the IFNAR1 receptor and ISFG3 activates the ISRE (8, 38). Inhibition of NF-κB could block LPS-stimulated IFNβ production and hence interfere with this positive feedback loop. However, our study points to a more direct effect because cells deficient in the β-chain of the IFNα/β receptor remain responsive to TLR4 and TLR3 signaling. Given our other data demonstrating that p65-depleted cells are impaired in the ISRE response to TLR4 but not TLR3 and that p65 could be detected in the LPS-activated ISRE-binding complex and could promote transactivation by IRF-3, we conclude that LPS directly induces a p65-IRF-3 complex for ISRE activation, whereas the poly(I-C) response is direct only toward IRF-3.

In conclusion, our study provides the first demonstration that p65 is absolutely required for activation of the ISRE by TLR4 but not TLR3. The emerging differences in signaling by TLR4 and TLR3 as well as other TLRs will continue to improve our understanding of the fine-tuning of the innate immune response.

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