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IRF-1-binding site in the first intron mediates interferon-γ-induced optineurin promoter activation

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ABSTRACT

Optineurin is an adaptor protein involved in signal transduction, membrane vesicle trafficking and autophagy. Optineurin expression is induced by cytokines. Previously we have shown that tumor necrosis factor-α activates optineurin promoter through NF-κB-binding site in the core promoter. However, this promoter was not activated by interferon-γ. Here, we report identification of a functional IRF-1-binding site in the first intron of human optineurin gene that mediates interferon-γ-induced activation of the promoter. Optineurin promoter, containing the contiguous intronic sequences with IRF-1 responsive sites, is strongly activated by IRF-1. Mutational inactivation of IRF-1 site resulted in loss of activation of the promoter by interferon-γ and also by IRF-1. We also show that IRF-1 cooperates with NF-κB to activate optineurin promoter. The synergistic effect of these two transcription factors (IRF-1 and NF-κB) may be involved in cooperative induction of optineurin promoter by interferon-γ and tumor necrosis factor-α.

1. Introduction

Optineurin is a multifunctional protein involved in diverse cellular processes including signal transduction, vesicular trafficking and autophagy [1–4]. It acts like an adaptor protein to coordinate various functions of different proteins interacting with it. It is a 577 amino acid protein with several coiled coil domains, a zinc finger domain, a leucine zipper domain, a ubiquitin-binding domain at its C-terminus and a recently identified LC3 (microtubule associated light chain 3)-binding domain at its N-terminus [4–8]. Presence of different domains in optineurin protein indicates towards its functional diversity. It interacts with Rab8, huntingtin and MyosinVI to coordinate movement of Rab8 vesicles on microtubular track [9–11]. It interacts with TBC1D17, a GTPase activating protein, to facilitate negative regulation of Rab8 by TBC1D17 [12]. Optineurin acts as an autophagy receptor to mediate cargo selective autophagy [4,13]. It regulates NF-κB signaling by facilitating deubiquitination of ubiquitinated RIP by CYLD [7,14]. Mutations in optineurin have been linked to normal tension glaucoma, a subtype of glaucoma in which the intraocular pressure remains within permitted range, and amyotrophic lateral sclerosis, a motor neuron disease [15,16]. Optineurin is also present in pathological structures associated with several neurodegenerative diseases [17].

Considering the diverse role played by optineurin, its tight regulation becomes imperative. Cytokines are important cellular molecules which act as stimuli to elicit response in the cell via signal transduction pathways to regulate protein levels. Optineurin expression has been shown to be induced by cytokines such as tumor necrosis factor-α (TNF-α) and interferons [6,18–20]. TNF-α is an inflammatory cytokine that has an important role in immune response, cancer, cell death and cell survival pathways [21]. The synthesis of TNF-α is induced by many different stimuli including interferons [22]. Interferons are part of the non-specific immune system which are induced at an early stage in response to viral infection and are released by cytolysis of the infected cells to protect surrounding cells [23]. IFN-γ is a type II interferon which exerts its effects through STAT1 and IRF-1 transcription factors to activate the expression of several genes [24–27].

Although induction of optineurin protein and mRNA by IFN-γ has been reported [6], the mechanism by which IFN-γ induces optineurin gene expression is not known. Previously it has been shown that optineurin promoter is activated by TNFα through NF-κB-binding site in the core promoter. NF-κB also contributes to basal optineurin promoter activity [18]. However, this promoter does not have any IRF-1 or STAT-1 binding sites and is not activated by IFN-γ. Here we have investigated the mechanism of regulation of optineurin gene expression by IFN-γ. We have identified IRF-1 as an activator of optineurin promoter which has a binding site in the first intron of human optineurin gene.

Abbreviations: IFN, interferon; IRF-1, interferon regulatory factor 1; LC3, microtubule associated light chain 3; TNF-α, tumor necrosis factor-α; STAT, signal transducer and activator of transcription; ChIP, chromatin immunoprecipitation.

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2. Materials and methods

2.1. Cell culture and transfections

A549 and HeLa cells were grown as monolayers in a humidified atmosphere of 5% CO_2 at 37 °C in DMEM (Dulbecco's minimal essential medium) containing 10% fetal calf serum. Transfections were performed using Lipofectamine Plus™ reagent (Invitrogen Life Technologies, CA, USA) according to the manufacturer's instructions. All the plasmids for transfection were prepared using Qiagen columns (Hilden, Germany). Recombinant human IFN-γ and TNF-α (Sigma, St. Louis, MO, USA and Calbiochem) were added for treatment wherever indicated at a final concentration of 100 ng/ml and 10 ng/ml, respectively.

2.2. Antibodies and expression vectors

Rabbit polyclonal anti-optineurin antibody was from Abcam (Cambridge, UK). Anti-IRF-1 antibody was obtained from Santa Cruz Biotechnology (CA, USA). Anti-GAPDH antibody was from Sigma (St. Louis, Missouri, USA). HRP conjugated anti-mouse and anti-rabbit antibodies were from Amersham (Piscataway, NJ, USA). IRF-1 expression plasmid has been described previously [28]. Plasmid for expressing p65 NF-κB was a kind gift by Dr. D. Karunagaran (Indian Institute of Technology, Chennai, India) and has been described [29].

2.3. Cloning of optineurin promoter with IRF-1 sites

The optineurin promoter was cloned from human genomic DNA by PCR amplification. The primers used were: forward, Opt-F, 5′-CCGCTCGAGACGGACAGCGAGGGTGGGTA-3′ and reverse Opt-R, 5′-CCCAAGCTTCTGAGGTCCTCACATTGCCTTA-3′. The sequence of this promoter matched completely with that present in the database (Homo sapiens chromosome 10 genomic contig, reference assembly, Ref. NT_077569.2 HS10_77618 from nucleotides 7504842 to 7505905). Deletion constructs (−136 to +844 and −136 to +785 bp; named D1 and D2) were made from the full length promoter (OPI) using reverse primers D1R 5′-CCC AAG CTT CGA TGT TTA CTT CCT GTA GCT TA-3′ and D2R 5′-CCC AAG CTT TAC AAC AGT TTT CCT GCT GGT GGA-3′ and same forward primer used for amplifying full length promoter. The optineurin promoter fragment and its deletion constructs were cloned into the pGL3-BASIC vector (Promega, Madison, WI, USA). IRF-1 site2 was mutated by site directed mutagenesis with the primers, forward 5′-ACT GTT TGT AAG GAT GAA ATG GAG GAT GAG GGC ATA GAA AAG TAA GGC AAT G-3′; reverse 5′-CAT TGC CTT ACT TTT CTA TGC CCT CAT CCT CCA TTT CAT CCT TAC AAA CAG T-3′ to create mOPI and confirmed by sequencing. Putative transcription factor binding sites were determined by using MatInspector software from Genomatics.

2.4. Western blot analysis

Cells were washed twice with PBS and lysed in 1× SDS sample buffer. Proteins were separated on 10% SDS poly-acrylamide gels and blotted onto nitrocellulose membranes, and processed further for western blotting as described earlier [28].

2.5. Reporter assays

Reporter assays were done for determining promoter activity as described [18]. Briefly, cells grown in 24-well plates were transfected with 100 ng of the required pGL3 construct, 50 ng of pCMV-SPORT β-gal (Invitrogen) and with the required amount of the other plasmids so as to keep the total amount of plasmids constant at 400 ng per well. Wherever required, cells were treated with IFN-γ or TNF-α after 6 h of transfection. Lysates were generally made 24 h post-transfection. Preparation of lysates and luciferase assays were carried out as per the instruction of manufacturer (Promega). Relative luciferase activities were calculated after normalizing with β-galactosidase enzyme activities.

2.6. Electrophoretic mobility shift assay

Nuclear extracts were prepared from control and IFN-γ treated Hela cells as described [18]. Electrophoretic mobility shift assay was carried out using radiolabeled synthetic oligonucleotide OPTN-IRF1-S2 essentially as described previously [18]. Protein–DNA complexes were separated in poly-acrylamide gel and detected by autoradiography.

2.7. ChIP assay

ChIP assay was done based on the protocol described in the information brochure of Cell Signaling Technology with certain modifications. Cells were fixed with formaldehyde, a reversible protein–DNA cross-linking agent that preserves the protein–DNA interactions occurring in the cell. Cells were then lysed and chromatin was harvested and fragmented using sonication. The chromatin was then subjected to immunoprecipitation using antibodies specific to IRF-1. After immunoprecipitation, the protein–DNA cross-links were reversed and the DNA was purified. The DNA sequences that were associated with the protein of interest (IRF-1), co-precipitated as part of the cross-linked chromatin complex and the relative amount of that DNA sequences (IRF-1-binding sites) was determined by PCR or quantitative real time

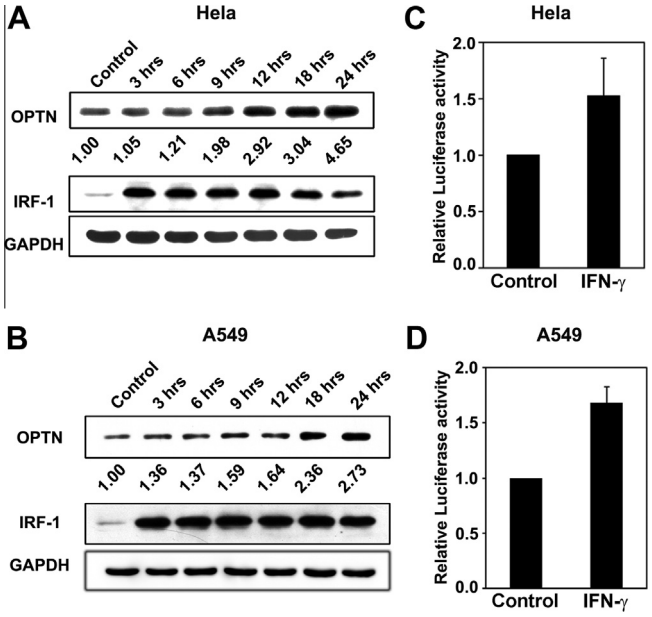

Fig. 1. Induction of optineurin expression and promoter activation by IFN-γ. Effect of IFN-γ on optineurin protein levels in HeLa (A) and A549 (B) cells. Cells were treated with IFN-γ for indicated time. Cell lysates were made, separated by SDS–PAGE and analyzed by western blotting using anti-optineurin, anti-IRF-1 and anti-GAPDH antibodies. The numbers indicate relative amount of optineurin protein. Hela (C) and A549 (D) cells grown in 24 well plates were transfected with 100 ng of optineurin promoter-reporter plasmid (full length construct pGL-OPI) along with pCMV-SPORT β-gal plasmid. After 6 h of transfection, IFN-γ was added. After another 18 h, cell lysates were prepared for reporter assays. Graph shows luciferase activities relative to untreated control after normalization with β-galactosidase activities from three separate experiments.

PCR using specific primers. PCR products were run on an agarose gel to facilitate quantification.

3. Results and discussion

3.1. IRF-1 activates optineurin promoter

IFN-γ has been shown to increase optineurin protein and mRNA level [6]. Initially we confirmed that treatment of A549, a human lung cancer cell line and HeLa cells with IFN-γ resulted in time dependent increase in optineurin protein as determined by western blot analysis (Fig. 1A and B). The optineurin protein level increased after 6 h of IFN-γ treatment in Hela cells as against 3 h in A549 cells and continued to increase up to 24 h. IRF-1 is one of the key targets and mediator of IFN-γ signaling [24]. In contrast to optineurin, IRF-1 protein level showed large increase after 3 h of IFN-γ treatment and remained at high level even after several hours (Fig. 1A,B). These results showed that upon IFN-γ treatment of A549 and HeLa cells IRF-1 is induced earlier than optineurin.

The optineurin promoter, which has been described earlier (−856 to +221) [18], was not activated by treatment of cells with IFN-γ (data not shown). Therefore, we attempted to identify potential STAT-1 and IRF-1 binding sites by bioinformatic analysis in upstream and downstream sequences. STAT-1 is a transcription factor which activates expression of a number of genes including IRF-1 [27]. Sequences 2 kb upstream of transcription start site and the first intron were analyzed for STAT-1 and IRF-1 binding sites. This analysis revealed two IRF-1 binding sites in the first intron that are located about 1 kb downstream of transcription start site. This region along with core promoter was amplified by PCR and cloned in pGL3 promoterless vector, upstream of luciferase reporter gene. The sequence of this promoter is shown in Fig. 2A. This promoter-reporter plasmid (OPI, Optineurin Promoter with IRF-1 sites) was transfected in Hela and A549 cells and the cells were treated with IFN-γ. The activity of this promoter was significantly increased upon treatment of cells with IFN-γ (Fig. 1C and D).

Upon treatment of cells with IFN-γ, one of the proteins induced is IRF-1 which is a transcriptional activator for many genes induced by IFN-γ [24,26,27]. We examined the effect of IRF-1 expression on optineurin promoter activity by co-transfection with IRF-1 expression plasmid. The optineurin promoter was strongly activated by expression of IRF-1, in a dose dependent manner (Fig. 2B). To our surprise, as low an amount of IRF-1 expression plasmid as 1 ng was able to activate the optineurin promoter significantly. However, this activation reached saturation when 25 ng or more of IRF-1 expression construct was used (Fig. 2B).

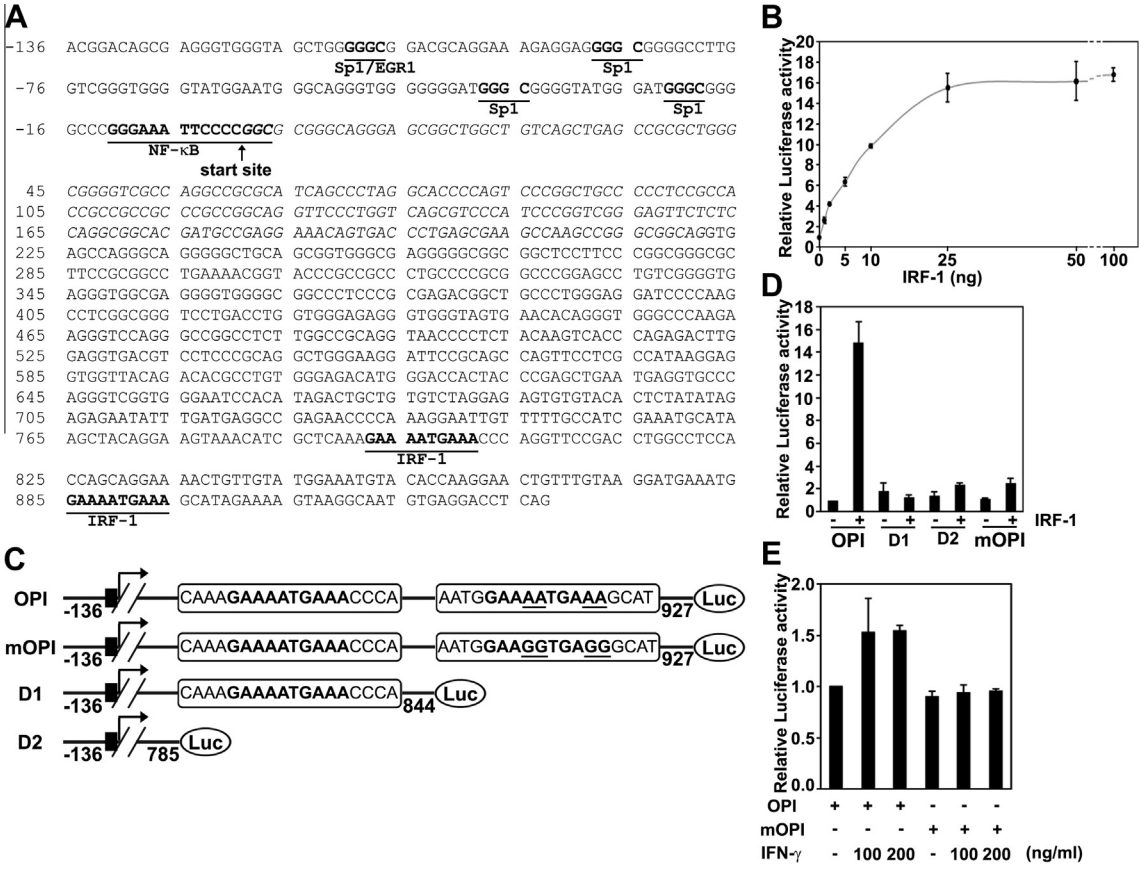

Fig. 2. Mutational analysis of optineurin promoter. (A) Nucleotide sequence of human optineurin promoter. Putative transcription factor binding sites for various transcription factors such as Sp1, NF-κB and IRF-1 are shown. First nucleotide of optineurin mRNA is taken as transcription start site (+1), indicated by an arrow. The first exon (shown in italics) is part of 5′-untranslated region of optineurin mRNA. (B) Hela cells grown in 24 well plates were transfected with 100 ng of optineurin promoter-reporter plasmid (full length construct pGL-OPI) along with pCMV-SPORT β-gal plasmid and varying amounts of IRF-1 expression plasmid. After 24 h, cell lysates were prepared for reporter assays. Graph shows average luciferase activities relative to control after normalizing with β-galactosidase enzyme activities from three independent experiments. (C) Schematic representation of various optineurin promoter-reporter constructs. Full length promoter (OPI), IRF-1 site mutant promoter (mOPI) and IRF-1-binding site deleted promoter 1 and 2 (D1 and D2), are shown. The nucleotide sequence of putative IRF-1 sites is shown and the nucleotides, which were mutated, are underlined. Black box indicates NF-κB site. (D) OPI, D1, D2 and mOPI were transfected along with 25 ng of IRF-1 expression plasmid and pCMV-SPORT-β gal in HeLa cells. Cell lysates were prepared after 24 h of transfection for reporter assays. (E) Wild type or mutant optineurin promoter constructs (OPI or mOPI) were transfected in HeLa cells, treated with 100 ng/ml or 200 ng/ml of IFN-γ after 6 h of transfection. After 24 h, cell lysates were prepared for reporter assays. Graphs show luciferase activities relative to untreated control promoter from three independent experiments.

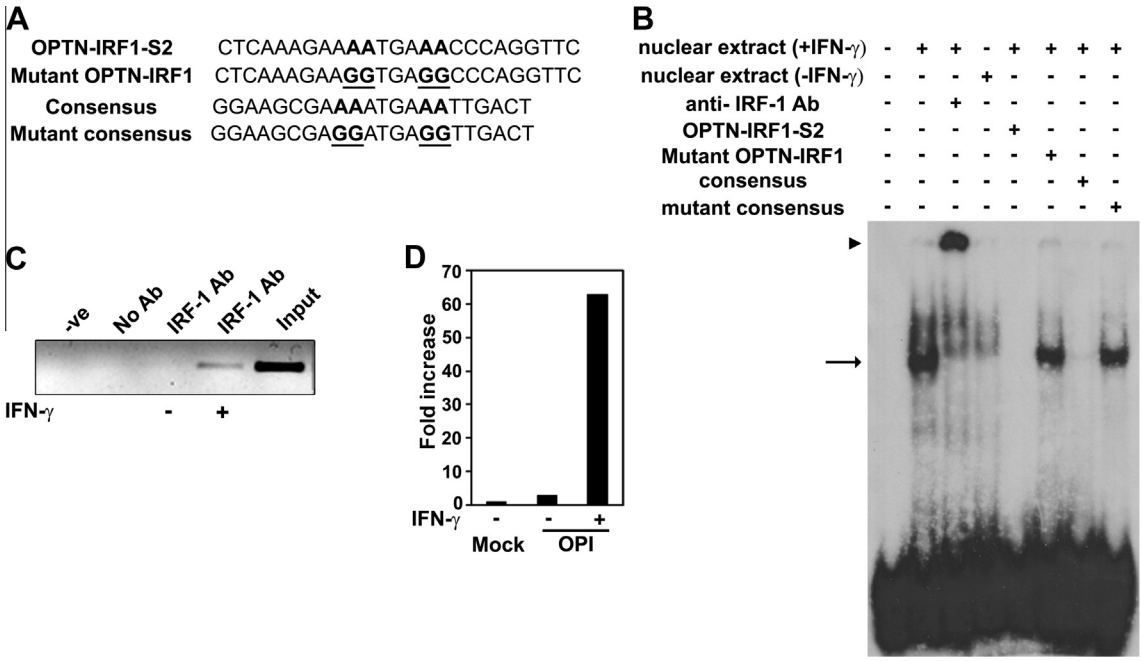

Fig. 3. IRF-1 binds to optineurin promoter. (A) Nucleotide sequence of oligonucleotides corresponding to putative IRF-1-binding site 2 (OPTN-IRF1-S2) in optineurin promoter, consensus IRF-1 binding oligonucleotide and the mutant oligonucleotides used for competition. (B) Electrophoretic mobility shift assay was carried out using radiolabeled OPTN-IRF1-S2 with nuclear extracts from Hela cells treated or untreated with IFN-γ. For competition 50-fold excess of unlabeled oligonucleotides was used. Arrow indicates shifted band and arrowhead indicates supershift. (C) Hela cells either treated or untreated with IFN-γ were subjected to ChIP assay. ChIP assay shows that anti-IRF-1 antibody immunoprecipitated IRF-1 binding elements of the optineurin gene. The PCR product was detected in cell nuclear extracts (input) as well as in IFNγ stimulated HeLa cells. (D) The immunoprecipitates from the ChIP assay were subjected to quantitative real time PCR and the graph shows the fold induction in the treated versus untreated samples.

3.2. Mutational analysis of optineurin promoter

To assess the role of putative IRF-1-binding sites in mediating the effect of IRF-1 in inducing optineurin promoter we carried out mutational analysis of the promoter. Two deletion mutants D1 and D2 were made. The D1 mutant lacks one of the IRF-1 binding sites whereas D2 lacks both of the IRF-1 binding sites (Fig. 2C). Both of these deletion constructs showed drastically reduced induction of promoter activity by IRF-1 (Fig. 2D). We also made a mutant promoter in which critical nucleotides in the core of IRF-1-binding site 2 were mutated (Fig. 2C). As compared with the wild type promoter, this mutant promoter showed drastically reduced activation by IRF-1 (Fig. 2D). Also, the mutant promoter, mOPI did not show any increase in activity upon treatment of cells with IFN-γ (Fig. 2E). These results suggest that IRF-1-binding site 2 is required for IFN-γ-induced activation of optineurin promoter but not for basal promoter activity.

To test whether IRF-1 binds directly to the IRF-1 responsive DNA sequences, we carried out electrophoretic mobility shift assay using a synthetic oligonucleotide corresponding to IRF-1 binding site 2 (Fig. 3A). The ^{32}P-labeled oligonucleotide was incubated with nuclear extract prepared from IFN-γ treated Hela cells. Binding to this nucleotide was seen (Fig. 3B) which was competed out with 50-fold excess of unlabeled self oligonucleotide and also with consensus IRF-1 binding nucleotide but not with mutant oligonucleotides in which IRF-1-binding site was mutated (Fig. 3B). Pre-incubation of the nuclear extract with anti-IRF-1 antibody resulted in supershift of the band (Fig. 3B). When this gel shift assay was carried out using nuclear extract prepared from control untreated cells, very little binding was seen (Fig. 3B). These results show that IRF-1 binds directly to the IRF-1-binding site 2 in the optineurin promoter.

To confirm whether IRF1 binds to the optineurin promoter in IFN-γ treated cells, we carried out a ChIP analysis. HeLa cells were either treated with IFN-γ or left untreated and then subjected to a ChIP assay with anti-IRF1 antibody. The chromatin immunoprecipitates were amplified by PCR, with specific primers to amplify IRF-1 binding elements. IRF-1 binding sites were seen to be amplified in cells treated with IFN-γ but not in untreated cells or negative controls (Fig. 3C). This result suggests that indeed IRF-1-binding region of optineurin promoter binds physically to IRF-1 in the cell. In addition to this we also performed quantitative real time PCR with the chromatin immunoprecipitates to validate our findings. The data showed that there was about 20-fold increase in IRF-1 binding sequences in IFN-γ treated cells compared to the untreated cells (Fig. 3D). Taken together, these results suggest that IFN-γ treatment stimulates transcription from optineurin promoter through its binding to IRF-1.

3.3. IRF-1 and NF-κB cooperate to induce optineurin promoter

Our earlier work has shown that TNF-α induces optineurin gene expression and its promoter activation through the transcription factor NF-κB [18]. Since the promoter cloned (in this study) possesses both IRF-1 as well as NF-κB binding sites, we investigated the possibility of interdependence between NF-κB and IRF-1 in the activation of optineurin promoter by TNF-α and IFN-γ. For this purpose, Hela cells were treated with IFN-γ alone, TNF-α alone or both IFN-γ and TNF-α and lysates were made for western blotting. Cells treated with both TNF-α and IFN-γ showed more induction of optineurin protein after 20 h of induction as compared to individual treatments (Fig. 4A). IRF-1 levels were also seen to be upregulated by treatment of cells with both TNF-α and IFN-γ as compared to IFN-γ alone (Fig. 4A). To test the possibility of cooperativity in promoter activation, the promoter-reporter construct was transfected in Hela cells and cells were treated with IFN-γ alone, TNF-α alone or the two together. The activity of this promoter was increased by 4.5-fold upon treatment of cells with IFN-γ and TNF-α

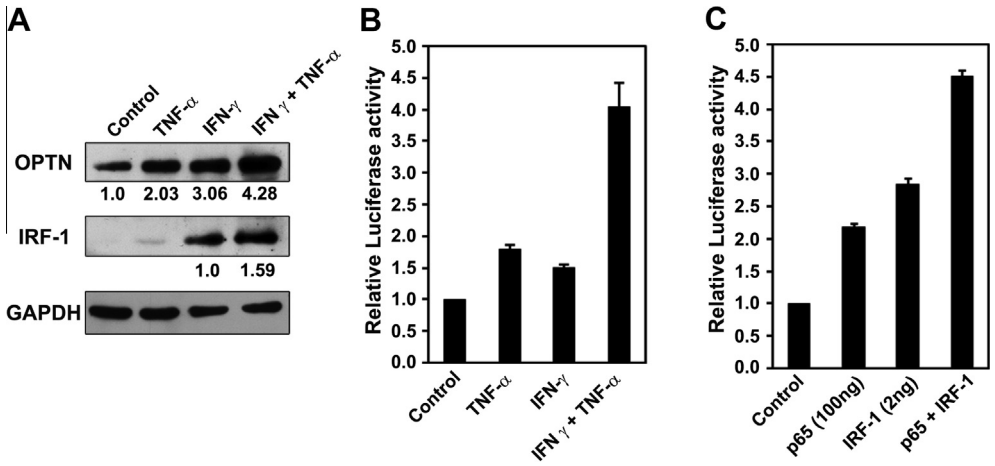

Fig. 4. TNF-α co-operates with IFN-γ in enhancing the optineurin promoter activity. (A) HeLa cells were treated with IFN-γ alone or TNF-α alone or both IFN-γ and TNF-α together for 20 h. Cell lysates were then prepared, separated by SDS–PAGE and analyzed by western blotting using antibodies against optineurin, IRF-1 and GAPDH. The numbers below the optineurin blot indicate relative amount of optineurin protein. (B) Full length optineurin promoter construct alongwith pCMV-SPORT-β gal plasmid was transfected in HeLa cells and after 6 h of transfection, cells were treated with IFN-γ alone or TNF-α alone or the two together. Cell lysates were prepared after 24 h of transfection for luciferase reporter assays. Luciferase activities relative to untreated control promoter are shown. (C) Hela cells were transfected with 100 ng of optineurin promoter-reporter plasmid (pGL-OPI) and pCMV-SPORT β-gal plasmid alongwith 100 ng of p65 NF-κB or 2 ng of IRF-1 expression plasmids or the two together. After 24 h, cell lysates were prepared for reporter assays.

in Hela cells as against 1.5-fold by IFN-γ and 1.8-fold by TNF-α (Fig. 4B). These results indicate towards cooperation between TNF-α and IFN-γ signaling for activation of the optineurin promoter resulting in enhanced protein level.

The cooperative effect of IFN-γ and TNF-α on optineurin promoter activity could be due to synergistic effect of IRF-1 and NF-κB. To examine this possibility, optineurin promoter was cotransfected with IRF-1 and p65 NF-κB expression plasmids alone or the two together and promoter activity was assessed. When put together, p65 and IRF-1 showed significantly increased promoter activity over IRF-1 alone (Fig. 4C). These results suggest that there exists a cooperativity between TNF-α and IFN-γ signaling via NF-κB and IRF-1, respectively, to induce optineurin promoter activity.

The results presented here show that IFN-γ, a critical component of immune defence, induces optineurin promoter activity. This induction of optineurin promoter is mediated by binding of IRF-1 to the DNA sequences in the first intron of optineurin gene. Our results show that IRF-1 is a strong activator of optineurin promoter activity. IRF-1 cooperates with NF-κB to activate optineurin promoter and the synergistic effect of these two transcription factors may be involved in inducing optineurin promoter activation in response to TNF-α and IFN-γ.

Acknowledgments

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