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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

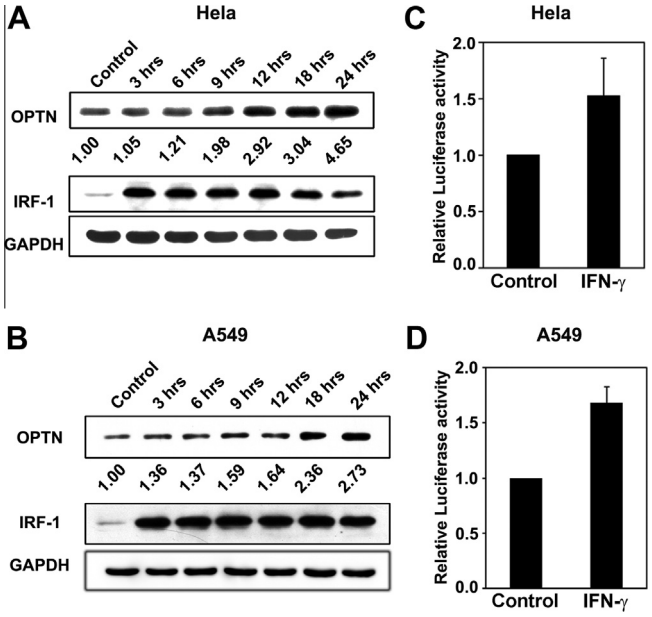

Fig. 1. Induction of optineurin expression and promoter activation by IFN-γ. Effect of IFN-γ on optineurin protein levels in HeLa (A) and A549 (B) cells. Cells were treated with IFN-γ for indicated time. Cell lysates were made, separated by SDS–PAGE and analyzed by western blotting using anti-optineurin, 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.