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Negative regulation of protein phosphatase 2Cβ by ISG15 conjugation

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Abstract ISG15, an interferon-upregulated ubiquitin-like protein, is covalently conjugated to various cellular proteins (ISGylation). In this study, we found that protein phosphatase 2Cβ (PP2Cβ), which functions in the nuclear factor κB (NF-κB) pathway via dephosphorylation of TGF-β-activated kinase, was ISGylated, and analysis by NF-κB luciferase reporter assay revealed that PP2Cβ activity was suppressed by co-expression of ISG15, UBE1L, and UbcH8. We determined the ISGylation sites of PP2Cβ and constructed its ISGylation-resistant mutant. In contrast to the wild type, this mutant suppressed the NF-κB pathway even in the presence of ISG15, UBE1L, and UbcH8. Thus, we propose that ISGylation negatively regulates PP2Cβ activity.

Keywords: Interferon-stimulated gene 15 kDa; Interferon; Ubiquitin; Protein phosphatase; Nuclear factor κB

1. Introduction

Interferon (IFN) is a pleiotropic cytokine that has an essential role in cellular antiviral response through inducing interferon-stimulated genes (ISGs) [1]. ISG15, one of the ISGs, is a ubiquitin-like protein that is covalently conjugated to various cellular proteins (ISGylation) upon interferon stimuli [2]. This protein functions as an antiviral protein against Sindbis virus and HIV-1 [3,4] and as a suppressor of the ubiquitin–proteasome system [5]. Although various target proteins for ISGylation have been identified by a proteomic approach [6–8] and a cascade of the protein ISGylation system has become clear [9–14], the biological consequences of ISGylation of target proteins have been studied in only a few cases [15–17].

Protein phosphatase 2Cβ (PP2Cβ) is an enzyme that belongs to the PP2C type protein phosphatase family and functions as a monomer [18]. PP2Cβ dephosphorylates and suppresses TGF-β-activated kinase (TAK1) and IκB kinase (IKK), both of which have essential roles in the nuclear factor κB (NF-κB) pathway, an important pathway functioning in innate immunity, adaptive immunity, and cancer [19–22].

We previously reported that ISGylation of UbcH6 and Ubc13 suppresses their ubiquitin E2 enzyme activities [16,17]. In a search for ISGylated proteins, we identified PP2Cβ as a target protein for ISGylation. In addition, we found that ISGylation of PP2Cβ suppresses the activity of PP2Cβ against TAK1-induced NF-κB activation.

2. Materials and methods

2.1. Cell culture and transfection

HeLa cells were cultured in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% heat-inactivated calf serum (Hyclone). Transfection was performed according to the standard calcium precipitation protocol.

2.2. Plasmid construction

The mammalian expression plasmids of ISG15, UBE1L, and UbcH8 were generated as described previously [17]. The open-reading frames of human PP2Cβ, TAK1, and TAK1-binding protein (TAB1) were amplified by PCR. Deletion and point mutants of PP2Cβ were generated by PCR. All constructs were verified by DNA sequencing. To generate mammalian expression plasmids of C-terminally and N-terminally Flag-tagged PP2Cβ, the PCR fragment was subcloned into pCI-neo-C3Flag and pCI-neo-3Flag vectors, respectively, which had been generated by inserting three repeats of Flag tag sequence into the pCI-neo mammalian expression vector (Promega). To generate the mammalian expression plasmids of TAK1 and TAB1, the PCR fragments were subcloned into the pCI-neo-2S and pCI-neo-5HA vectors that had been generated by inserting oligonucleotides encoding two repeats of S peptide sequence and five repeats of HA tag sequence, respectively, into the pCI-neo mammalian expression vector.

2.3. Immunoprecipitation and Western blotting

HeLa cells that had been transiently transfected with indicated plasmids and cultured for 24–30 h were lysed with RIPA buffer containing 50 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride and 5 mM *N*-ethylmaleimide, and the supernatant of the cell lysate was subjected to immunoprecipitation and then to Western blotting with various antibodies as described previously [16,17] except for anti-PP2Cβ [19] and anti-IκBα (BD Biosciences) antibodies. The effect of IFN treatment was analyzed as described previously [17] except for HeLa cells and lysis buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 5 mM *N*-ethylmaleimide.

2.4. Reporter gene assay

HeLa cells were transfected with the pNF-κB-Luc reporter plasmid (BD Biosciences) and indicated plasmids, together with pRL-TK (Promega) for normalizing transfection efficiency. Twenty-four hours after transfection, cells were lysed and luciferase activity was measured by using a Dual-Luciferase Reporter Assay System (Promega) and an AB-2000 luminescencer-PSN (Atto, Tokyo, Japan). The same experiments were repeated three times.

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Abbreviations: ISG15, interferon-stimulated gene 15 kDa; E1, ubiquitin-activating enzyme; E2, ubiquitin-conjugating enzyme; PP2Cβ, protein phosphatase 2Cβ; TAK1, TGF-β-activated kinase; TAB1, TAK1-binding protein; NF-κB, nuclear factor κB; IκB, inhibitor of NF-κB; IKK, IκB kinase; IFN, interferon

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3. Results and discussion

3.1. PP2Cβ is modified with ISG15

To identify proteins modified with ISG15, we carried out a proteomic analysis of ISGylated proteins. We expressed Flag-tagged ISG15, S-tagged UBE1L (E1), and S-tagged UbcH8 (E2) in HeLa cells, and ISGylated proteins were isolated by immunoprecipitation with anti-Flag antibody and subjected to peptide mass fingerprinting. We identified several novel ISGylated proteins (data not shown; data to be presented elsewhere). Among them, we focused on PP2Cβ because PP2Cβ dephosphorylates and suppresses TAK1 and IKK, kinases both functioning in the NF-κB pathway [19,20].

To confirm ISGylation of PP2Cβ, we expressed Flag-tagged PP2Cβ together with T7-tagged ISG15, S-tagged UBE1L and S-tagged UbcH8 in HeLa cells, and the extract of transfected cells was subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-T7 tag and anti-Flag tag antibodies (Fig. 1). Two bands (the molecular masses of 88 and 110 kDa) with slower mobilities than that of intact PP2Cβ (67 kDa) were detected by anti-Flag tag antibody in UBE1L- and UbcH8-dependent manners and these bands were also detected by anti-T7 tag antibody: The above difference in molecular masses between modified and intact ones is reasonably thought to be due to ISGylation. These results strongly suggest that PP2Cβ is covalently modified with ISG15, the conjugation being mediated by UBE1L and UbcH8. It should be noted that ISGylation of various proteins was detected by overexpression of UBE1L in the presence of ISG15 and was further enhanced by co-expression of UBE1L and UbcH8 (Fig. 1, left panel): These results are consistent with the previous report [11].

Next, to confirm that PP2Cβ is ISGylated in response to IFN signal, HeLa cells that had expressed Flag-tagged PP2Cβ were treated with IFNβ. The cell extract was subjected to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-ISG15 antibodies (Fig. 2A). In another experiment (Fig. 2B), the extract of HeLa cells that had been treated with IFNβ was subjected to immunoprecipitation with anti-PP2Cβ antibody and then to Western blotting with anti-PP2Cβ and anti-ISG15 antibodies. In both experiments, two bands due to ISGylated PP2Cβ were detected only in the case of IFN treatment, indicating that PP2Cβ is ISGylated in response to IFN signal under physiological conditions. ISGylation of various proteins was also detected only in the case of IFN treatment (Fig. 2A and B, right panels). It should be noted that the molecular mass (67 kDa) of exogenously expressed Flag-tagged PP2Cβ isoform 1, the longest isoform of human PP2Cβ [23] and the isoform used in this study, is different from that (42 kDa) of endogenous PP2Cβ isoform 2, the most abundant isoform in HeLa cells [24].

3.2. PP2Cβ activity is suppressed by addition of a protein ISGylation system

It has been reported that PP2Cβ dephosphorylates and suppresses TAK1 [19], which functions in the NF-κB pathway together with TAB1 [25]. We therefore carried out an experiment to determine whether a protein ISGylation system modulates PP2Cβ activity against TAK1/TAB1-induced NF-κB activation. HeLa cells were co-transfected with plasmids of HA-tagged TAK1 and TAB1, Flag-tagged PP2Cβ, and ISGylation system (T7-tagged ISG15, S-tagged UBE1L, and S-tagged UbcH8), together with NF-κB luciferase reporter plasmid, and luciferase activity was measured (Fig. 3). PP2Cβ suppressed TAK1/TAB1-induced NF-κB activation in the absence of the ISGylation system but not in the presence of the ISGylation system. It should be noted that TAB1 alone has little effect [25]. These results suggest that ISGylation of PP2Cβ suppresses the activity of PP2Cβ against TAK1/TAB1-induced NF-κB activation.

3.3. PP2Cβ is modified with ISG15 at least through Lys12 and Lys142

To confirm the above assumption, it is necessary to construct an ISGylation-resistant mutant of PP2Cβ. Since PP2Cβ is expected to be ISGylated through at least two Lys residues

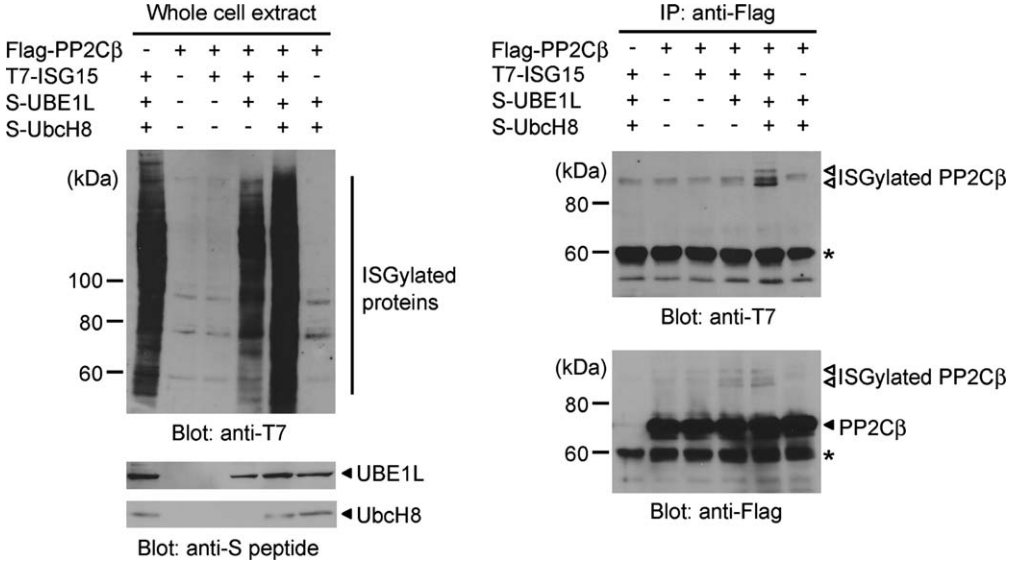

Fig. 1. PP2Cβ is covalently modified with ISG15. The extracts of HeLa cells that had been transiently transfected with indicated plasmids were subjected to Western blotting with anti-T7 tag and anti-S peptide antibodies (left panel) or to immunoprecipitation with anti-Flag tag antibody and then to Western blotting with anti-Flag tag and anti-T7 tag antibodies (right panel). The ISGylated PP2Cβ is indicated by an open arrowhead. Non-specific bands are indicated by asterisks.