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IKKβ specifically binds to P16 and phosphorylates Ser8 of P16

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ABSTRACT

In spite of its central roles in cell cycle progression, senescence, and aging, knowledge about the posttranslational regulation of P16 (also known as INK4A and MTS1) remains limited. While it has been reported that P16 could be phosphorylated at Ser7, Ser8, Ser140, and Ser152, the corresponding kinases have not been identified yet. Here we report that IKKβ, a primary kinase for IκBα phosphorylation, is involved in P16 phosphorylation. Immunoprecipitation and kinase assays showed that IKKβ specifically binds to P16 and phosphorylates P16 at Ser8 in WI38 cells. Biochemical characterization of phosphomimetic Ser → Glu P16 mutants demonstrated that phosphorylation at Ser8 of P16 brings about a significant loss of its cyclin-dependent kinase (CDK) 4-inhibitory activity while P16 retains structurally and functionally intact upon phosphorylation at Ser7, Ser140, and Ser152. Our results reveal the novel role of IKKβ in P16 phosphorylation and broaden our understanding of the regulation of P16.

Introduction

P16, also designated INK4A and MTS1, is one of the most extensively studied proteins in the past decades due to its critical roles in cell cycle progression, cellular senescence, and the development of human cancers. At the G_1-to-S transition, P16 specifically inhibits cyclin-dependent kinases (CDK) 4- and 6-mediated phosphorylation of pRb, the retinoblastoma susceptible gene product, thus sequestering the transcription factors of E2Fs in incompetent pRb/E2F complexes and consequently blocking cell cycle progression [1]. It has also been demonstrated that elevated expression of P16 caused by certain oncogenes, DNA damage response, or aging triggers and accelerates cell senescence [2,3]. Moreover, while genetic inactivation of the *p16* gene (*CDKN2A*) by deletion, methylation, and point mutations has been found in a significant fraction (close to 50%) of all human cancers [4], over-expression of P16 at both mRNA and protein levels is associated with poor prognosis for cancers including neuroblastoma, cervical, ovarian, breast, prostate tumors, and oral cancers [5], indicating that the cellular level of P16 protein is critical for its functioning. In comparison with genetic and transcriptional regulation of the *p16* gene, posttranslational regulation of P16 has been understudied. It has been reported that P16 could be phosphorylated in human fibroblast cells at Ser7, Ser8, Ser140, and Ser152 [6], all of which are located at the flexible N- and C-termini and do not directly contact CDK4 [7]. Such phosphorylation is potentially important since mutations involving these four residues have been found in familial and sporadic melanomas [6]. Nonetheless, the kinases responsible for P16 phosphorylation as well as the functional and structural effect upon P16 phosphorylation remain unknown.

Recently, it has been reported that there are striking functional and structural similarities between P16 and IκBα, a well-known inhibitor of NF-κB [8–10]. On one hand, P16 and IκBα compete with each other for binding to CDK4 and NF-κB, and such binding specifically inhibits the activities of both CDK4 and NF-κB [8,10]. On the other hand, while P16 and IκBα are composed of 4 and 6 ankyrin repeats (ARs), respectively, the CDK4-binding domain of IκBα is located at the four N-terminal ARs, and the structures of these four ARs in P16 and IκBα are almost superimposable, especially in the helical regions where most of contacts with their target proteins are located [9]. More interestingly, both P16 and IκBα have flexible N-termini harboring two phosphorylation sites, Ser7/Ser8 in P16 [6] and Ser32/Ser36 in IκBα [11]. These findings arguably lead to a postulation that P16 and IκBα, especially their N-termini, may be similar in phosphorylation, i.e. kinases involved in IκBα phosphorylation may function in the regulation of P16.

In the present study, we demonstrated that IKKβ, an IκBα-specific kinase [11], physically associates with P16 in vivo, and the resultant phosphorylation at Ser8 of P16 significantly impairs the CDK4-inhibitory activity of P16.

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Materials and methods

Protein expression and purification. The cloning, expression, and purification of human P16, IκBα1–214, and Yar 1 have been described previously [8]. Briefly, all P16, IκBα1–214 and Yar 1 proteins including WT and different mutants were expressed in *Escherichia coli* BL21 Codon Plus (Novagen) as Glutathione-*S*-transferase (GST)-fusion proteins and purified using reduced Glutathione resin (Sigma). After removal of the GST tag by Thrombin (for P16; Sigma) or Prescission protease (for IκBα1–214 and Yar 1; Amersham), proteins were further purified on a gel filtration column. All P16 mutants were generated using PCR-based site-directed mutagenesis (Stratagene), and were expressed and purified as P16 wild type (WT).

Cell culturing, immunoprecipitation (IP) and Western blot (WB). U2OS ($p16^{-/-}$) and WI38 ($p16^{+/+}$) cells were purchased from the American Type Culture Collection and cultured in a 5% CO_2 humidified atmosphere in Advanced McCoy's 5A and Advanced MEM containing 7% fetal bovine serum (FBS, Invitrogen), respectively. WI38 cells were used at passage 3–5. Cells were lysed in the non-denaturing lysis buffer (20 mM Tris–HCl, pH 7.4, 0.1% NP-40, 250 mM NaCl, 5 mM EDTA, 20 mM NaF, 2 mM Na_3VO_4, 1 mM DTT, and 200 μg/ml Sigma P8340 protease inhibitor cocktail). After incubation on ice for 10 min, the cells were clarified by centrifugation at 4 °C, 20,000*g* for 15 min. The supernatant was then transferred to a clean tube and the protein concentration was determined using a BCA protein assay (Pierce). For protein expression analyses, 50 μg of cell lysates were subjected to SDS–PAGE and western blot to evaluate protein expression using indicated antibodies: IKKβ, sc-56918 (Santa Cruz Biotechnologies); P16, Cat. #554070 (PharMingen); β-actin, sc-56459 (Santa Cruz Biotechnologies). Blots were visualized using the Pico Western Chemiluminescent system (Pierce). For immunoprecipitation (IP) analyses, 400 μg of cell lysates were immunoprecipitated with the afore-mentioned antibodies or a combination of normal mouse serum and rabbit serum (Jackson Immunoresearch Laboratories) [6]. Antibody complexes were captured with 70–100 μl lysis buffer-pretreated protein G-Sepharose (Amersham). Immunoprecipitates were washed three times using the lysis buffer and subjected to further analyses; cell lysates with the removal of immunoprecipitates were used in the *in vitro* P16 phosphorylation assay as described below. Since there is no endogenous P16 in U2OS, 2 μg of recombinant P16 protein was added into 400 μg of U2OS cell lysate, and after incubation at 4 °C for 4 h, the mixture was subjected to immunoprecipitation using anti-IKKβ antibody as described above.

In vitro phosphorylation of P16. Reaction mixtures containing 0.1 μg of recombinant IKKβ (Invitrogen), 2.0 μg of P16 proteins, and 5 μCi [γ-^{32}P] ATP in a total volume of 15 μl of the kinase buffer (50 mM HEPES, 10 mM $MgCl_2$, 2.5 mM EGTA, 0.1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerolphosphate, 1 mM DTT, 0.2 mM AEBSF, 2.5 mg/ml leupeptin, and 2.5 mg/ml aprotinin) were incubated at 30 °C for 20 min. Subsequently, the mixtures were subjected to SDS–PAGE and radio autography. Cell lysate-mediated P16 phosphorylation was evaluated similarly except that each reaction contained 10 μg of U2OS or WI38 cell lysate, or 10 μg of IKKβ-depleted cell lysate, and the incubation at 30 °C lasted for 45 min. IκBα1–214, truncated IκBα containing Ser32 and Ser36 for IKKβ phosphorylation [8,11], was used as positive control, while Yar 1, a yeast AR protein of 200 amino acid residues was used as negative control [8].

In vitro inhibition of P16 on CDK4. The *in vitro* CDK4 activity assay was performed as previously described [7,8]. Briefly, each reaction mixture contains about 0.2 μg of recombinant CDK4/cyclin D2 holoenzyme and varying concentrations of P16 in 15 μl of the afore-mentioned kinase buffer. After incubation at 30 °C for 30 min, 50 ng of GST-Rb791–928 and 5 μCi [γ-^{32}P] ATP were added in the reaction mixture and after incubation at 30 °C for another 15 min, proteins in the reaction mixture were separated by SDS–PAGE, and the incorporation of ^{32}P into GST-Rb791–928 was quantitatively evaluated using a PhosphorImager (Molecular Dynamics). The IC_{50} value was defined as the concentration of a kinase inhibitor required to achieve 50% of the maximal inhibition of CDK4 [7], and measurements were performed in triplicate.

Circular dichroism (CD) analyses. Samples containing 7.5–10.0 μM proteins in 20 mM sodium borate–40 μM DTT buffer (pH 7.4) were incubated with different amounts of guanidinium chloride (GdnHCl, in a stock solution of 8.5 M) on ice overnight and then equilibrated at 25 °C just prior to CD analysis [9]. The rotation at 222 nm was measured on an AVIV far-UV spectropolarimeter using a quartz microcell (Helma) of 0.1 cm light pass length, and the exact concentrations of GdnHCl were determined using the refractive index. For each sample, three scans were averaged, and the free energy of protein denaturation in aqueous condition was obtained on the basis of two-state approximation [9]. Heat-induced unfolding experiments were performed using 5.0 μM proteins with a 1-nm bandwidth and a 10-s response time. Thermal melting spectra were recorded at 222 nm by heating from 5 to 65 °C at the rate of 1 °C/min and a 1 °C interval followed by cooling down to 5 °C at the same rate. T_m was defined as the temperature at the midpoint of transition [9].

NMR analyses. NMR samples contained 0.4 mM protein, 5 mM HEPES, 1 mM DTT, 5 μM EDTA in 90% H_2O/10% D_2O at pH 7.4 [7]. The 1D ^{1}H NMR and 2D ^{1}H-homonulcear NOESY experiments (200 ms mixing time) were performed at 20 °C on a Bruker DMX_600 spectrometer, which was equipped with a 5 mm triple-resonance probe and three-axis gradient coil. Data were processed with XWINNMR 3.5 (Bruker).

Results

IKKβ specifically interacts with P16 and phosphorylates Ser8 of P16

IKKβ is the primary kinase to phosphorylate Ser32 and Ser36 of IκBα thus triggering the release of NF-κB from the inactive NF-κB/IκBα complex [11]. The activation of IKKβ, as a result of inflammatory cytokine signaling, infectious agents, and DNA damage, has been regarded as a predominant pathway in regulating the activity of NF-κB [11]. To explore the potential interaction between IKKβ and P16, we first investigated the potential P16/IKKβ interaction through immunoprecipitations. WI38 ($p16^{+/+}$) cells were chosen for this study mainly due to the fact that these cells have endogenous P16 as well as P16-phosphorylating activities as demonstrated in previous studies [6]. In contrast, U2OS, a *p16*-null cell line was used as a control. As shown in Fig. 1A, IKKβ was expressed in both WI38 and U2OS cells while P16 was only present in WI38. In the immunoprecipitation assay using anti-P16 antibody, IKKβ was present only in the immunoprecipitates from WI38 cells (Fig. 1B). Similarly, in immunocipitation using anti-IKKβ antibody, P16 was detected in the immunoprecipitates from WI38, not U2OS (Fig. 1C). These results indicate that P16 and IKKβ interact with each other in WI38 cells. As control, such interaction was not observed in U2OS cells simply due to the absence of endogenous P16. Indeed, as shown in Fig. 1C, when exogenous P16 was added to the lysates of U2OS cells, P16 was detected in the immunoprecipitates (using anti-IKKβ), implying that exogenous P16 is able to physically associate with cellular IKKβ.

We then examined the potential phosphorylation of IKKβ on P16 using an *in vitro* assay. As shown in Fig. 2A, in a reaction mixture containing recombinant IKKβ and P16, P16 was phosphorylated as the positive control, IκBα1–214, a truncated form of IκBα containing the intact N-terminus and four ARs [8,11]. In contrast, no