Distinct B subunits of PP2A regulate the NF-κB signalling pathway through dephosphorylation of IKKβ, IκBα and RelA

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PP2A is composed of a scaffolding subunit (A), a catalytic subunit (C) and a regulatory subunit (B) that is classified into four families including B, B’, B” and B’’/striatin. Here, we found that a distinct PP2A complex regulates NF-κB signalling by dephosphorylation of IKKβ, IκBα and RelA/p65. The PP2A core enzyme AC dimer and the holoenzyme AB000C trimer dephosphorylate IKKβ, IκBα and RelA, whereas the ABC trimer dephosphorylates IκBα but not IKKβ and RelA in cells. In contrast, AB’C and AB’’C trimers have little effect on dephosphorylation of these signalling proteins. These results suggest that different forms of PP2A regulate NF-κB pathway signalling through multiple steps each in a different manner, thereby finely tuning NF-κB- and IKKβ-mediated cellular responses.

Keywords: NF-kappa B; protein phosphatase; protein phosphatase 2 A (PP2A)

Nuclear factor kappa B (NF-κB) is a critical transcription factor that regulates many cellular and organismal processes including immune and inflammatory responses, cellular growth and cell survival. The NF-κB signalling pathway is regulated by the phosphorylation of several proteins including inhibitor of kappa B (IκB) kinase β (IKKβ), an inhibitory protein IκBα and an essential subunit of NF-κB RelA/p65 [1–3]. Binding of cytokines such as TNF-α to respective receptors leads to the conjugation of ubiquitin to several signalling components and recruits adaptor proteins and kinases [3–6]. These chains of reaction induce IKKβ activation through phosphorylation of two critical serine residues, Ser177 and Ser181, at its activation loop [7,8]. IKKβ in turn phosphorylates IκBα at the N-terminal serines, Ser32 and Ser36, which leads to ubiquitination at lysine residues Lys21 and Lys22 and IκBα degradation by the ubiquitin-proteasome system. These reactions result in the nuclear translocation of NF-κB and binding to its cognate κB sites in the promoters/enhancers of its target genes. IKKβ also phosphorylates serine residues, Ser468 and Ser536, in the C-terminal transactivation domain of RelA and promotes gene expression through the association of RelA with a transcriptional coactivator, CREB-binding protein [9–11].

Aberrant activation of NF-κB is linked to various diseases such as inflammatory disorders and cancer. Thus, there are numerous regulatory mechanisms at multiple levels to ensure the tight control of NF-κB activity [1–6]. In particular, dephosphorylation of its protein represents the most important mechanism to downregulate NF-κB activity. There are three families of protein serine/threonine phosphatases: protein phosphatases (PPPs), metal-dependent protein phosphatases (PPMs) and protein phosphatases.
and DxDxT phosphatases [12,13]. PPP constitutes the largest family, containing several members including protein phosphatase 1 (PP1), PP2A, PP2B and PP5, whereas the PPM family includes PP2C. Several of these, such as PP1 [14], PP2A [7,15,16], PP2C [17] and PP5 [18], are required for dephosphorylation of the activation loop serines in IKKβ, which is a critical step to downregulate NF-κB. In addition, protein phosphatases dephosphorylate downstream signalling proteins as well. Specifically, it has been reported that PP2B dephosphorylates IκBα [19] and PP2A [20] and PP2C [21] dephosphorylate RelA.

Among these phosphatases, PP2A is the most abundant, constituting approximately 1% of total cellular proteins [22–24]. PP2A is a heterotrimeric complex consisting of a scaffolding (A), regulatory (B) and catalytic (C) subunit. The A and C subunits each are comprised of two possible variants, α and β. The monomeric C subunit is unstable and requires binding to the A subunit to exist as a stable core enzyme AC dimer. The B subunit family consists of four classes including B (B56/PR93/PR110)/striatin (Strn). In turn, there are four isoforms of the B class including α, β, γ and δ, five of the B’ class including α, β, γ, δ and ε, three of the B’’ class including α, β and γ, and three isoforms of the B’’’/Strn class including Strn, Strn3 and Strn4. These B subunits associate with the AC dimer to form the holoenzyme trimer. Crystal structure analysis revealed that the B subunits bind proximal to the C subunit active site in the holoenzyme trimer and determine the specificity for substrate proteins [25,26].

A previous RNAi screen revealed that PP2A plays an important role in the regulation of NF-κB signalling and identified the core enzyme subunits, Aα, Bβ, Cα and Cβ, as negative regulators of the NF-κB signalling pathway in mouse astrocytes [16]. However, this screen did not clarify whether the B subunit is involved in regulation of NF-κB activity, nor identify the specific B subunits regulating each NF-κB signalling step. It is plausible that, as cells usually express multiple isoforms of each B family protein, this failure was a result of complementation with other isoforms of the same family following the simple RNAi screen. In this study, we expressed B subunits together with A and C subunits in cells and investigated the role of these subunits on TNF-α-, IKKβ- and RelA-induced NF-κB activation. This assay revealed that distinct B subunits regulate specific steps of NF-κB signalling. Dephosphorylation of IKKβ and RelA is mediated by the AC dimer and AB’’C trimer, whereas dephosphorylation of 1xβα is mediated by the AC dimer, ABC trimer and AB’’’C trimer.

Materials and methods

Cell cultures, plasmids and transfection
cDNA-encoding Aα, Bβ, Cα, Cβ, Bα, Bβ, Bγ, Bδ, Bα, Bβ, Bγ, Bδ, Bα, Bβ, Strn and Strn3 subunits of PP2A were amplified from a human cDNA library by PCR. The cDNAs were inserted into pRKH-HA and pRKH-Flag expression vectors. Expression plasmids encoding IKKβ, kinase-negative IKKβ mutant (IKKβ/KKN) and 1xβ2 have been reported previously [27]. Constitutive active IKKβEE mutant of which phosphorylation sites Ser177/181 in the activation loop were exchanged to Glu, the ubiquitination-resistant 1xβ2RR mutant of which ubiquitination sites lysine residues Lys21/22 were exchanged to Arg, and the phosphatase-inactive CαD85N mutant of which Asp 85 was exchanged to Gln were generated using the KOD-Plus Mutagenesis kit (TOYOBO). Plasmids were transfected into GP2-293 cells in Opti-MEM (Invitrogen) using Lipofectamine Plus (Invitrogen) following the manufacturer’s instructions. GP2-293 cells were obtained from Clontech. Cells were cultured in DMEM supplemented with 10% FBS containing 2 mM L-glutamine at 37 °C.

Luciferase assay

Nuclear factor kappa B activity was estimated by Dual-Luciferase Reporter Assay System (Promega) using pNF-kB and pRL-TK Luciferase reporter plasmids. After transfection of 0.01 µg NF-kB reporter plasmids with or without 0.01 µg expression plasmids of various PP2A subunits, IKKβ, 1xβα and/or RelA into GP2-293 cells in collagen-coated 96-well dishes, cells were incubated in the presence or absence of 50 ng·mL⁻¹ TNF-α for 18 h. After lysing cells with buffer from the assay system, luciferase activity was analysed following the manufacturer’s instructions.

Immunoblotting

GP2-293 cells in collagen-coated 12-well dishes were transfected with 0.2 µg expression plasmids of various PP2A subunits, IKKβ, 1xβ2 and/or RelA. After 24-h transfection, cells were washed with PBS and solubilized with buffer A consisting of 20 mM Tris/Cl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 10 mM MgCl2, 60 mM β-glycerophosphate, 1 mM Na2VO4, 1 mM 4-aminophenyl methyl sulfonyl fluoride, 50 KIU·mL⁻¹ aprotinin, 20 µg·mL⁻¹ pepstatin, 20 µg·mL⁻¹ leupeptin, 2 mM DTT and 1% Triton X-100. After centrifugation at 16 000 × g for 20 min at 4 °C, the supernatants were used as cell lysates. The cell lysates were subjected to SDS-PAGE and then gel-separated proteins were transferred to PVDF membranes (Millipore) and subjected to immunoblotting using the SuperSignal West Pico Chemiluminescence System (Pierce). Antibodies used were as follows: anti-Flag (M2) (Sigma, F-3165), anti-HA...
In vitro dephosphorylation assay by PP2A

For the dephosphorylation assay of IKKβ, GP2-293 cells in 10-cm dishes were transfected with expression plasmids encoding Flag-IKKβ, and then phosphorylated IKKβ was recovered from cell lysates via anti-Flag (M2) agarose beads (Sigma). IKKβ proteins were eluted in a buffer containing 20 mM Tris/Cl, pH 7.5, 150 mM NaCl, 2 mM DTT, and 1% Triton X-100 and 3 Units (Millipore). For the dephosphorylation assay of IKKα and anti-Flag-agarose beads with ultra-free Centrifugal Filter Units in the previously described buffer.

Distinct B subunits of PP2A regulate NF-κB signalling

To investigate the involvement of the PP2A in regulation of NF-κB signalling, we constructed expression plasmids encoding each subunit of the B, B’α, B’β and B’/Strn family (Fig. 2A). Among isoforms of B’α and B’β/Strn families, we used expression plasmids encoding B’β for the B’β family, along with Strn and Strn3 for the B’/Strn family, as B’α, B’γ and B’/Strn4 hardly express in cells. Cells were transfected with NF-κB reporter plasmids and plasmids encoding Aα, Cα and each B subunit, and NF-κB activity was investigated following TNF-α treatment by luciferase assay. Expression of the AC holoenzyme, the ABC holoenzyme including A(Bα)C, A(Bβ)C, A(Bγ)C and A(Bδ) C, and the AB’’C holoenzyme including A(Strn)C and A(Strn3)C suppressed TNF-α-induced NF-κB activity, whereas the AB’C holoenzyme including A(B’α)C, A(B’β)C, A(B’γ)C, A(B’δ)C and A(B’ε)C and the AB’’C holoenzyme including A(B’’β)C had little effect on TNF-α-induced NF-κB activation (Fig. 2B). Then, we investigated the effects of distinct B subunits on NF-κB signalling. NF-κB activity was analysed in cells transfected with plasmids encoding IKKβ or IKKβEE together with Aα, Cα and each B subunit. A luciferase assay demonstrated that expression of AC, ABC and AB’’C attenuated IKKβ- and IKKβEE-induced NF-κB activity, whereas AB’C and AB’’C had little effect on NF-κB activation (Fig. 2C,D). These results suggest that AC, ABC and AB’’C suppress NF-κB activity downstream of IKKβ.

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subunit, and phosphorylation of the activation loop was investigated by immunoblotting. Expression of the AC core enzyme markedly suppressed IKK\(\beta\) phosphorylation in a phosphatase activity-dependent manner (Fig. 3A). The AC core enzyme and the AB\(000\)C holoenzyme markedly suppressed IKK\(\beta\) phosphorylation, whereas three types of holoenzyme including ABC, AB\(C\) and AB\(00\)C had little effect (Fig. 3B). These lines of evidence indicate that AC and AB\(000\)C attenuate TNF-\(\alpha\)-induced NF-\(\kappa\)B activity, at least in part, through dephosphorylation of IKK\(\beta\).

To reveal the effects of each B subunit on the NF-\(\kappa\)B/IkB\(\alpha\) complex, we transfected cells with expression plasmids encoding IkB\(\alpha\)RR, RelA and IKK\(\beta\)EE,
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Fig. 3. PP2A enzyme AC and AB′′′C suppress IKKβ and RelA phosphorylation, and AC, ABC and AB′′′C suppress IκBα phosphorylation in vivo. (A) GP2-293 cells were transfected with plasmids encoding IKKβ and PP2A plasmids encoding Aα and Cα or phosphatase-inactive CαD85N. (B) Cells were transfected with plasmids encoding IKKβ and PP2A plasmids encoding Aα, Cα and each subunit of B, B′, B″ and B′′′/Strn families. (C) Cells were transfected with plasmids encoding IKKβ, IκBα and RelA and PP2A plasmids encoding Aα and Cα or CαD85N. (D) Cells were transfected with plasmids encoding IKKβ, IκBα and RelA and PP2A plasmids encoding Aα, Cα and each subunit of B, B′, B″ and B′′′/Strn families. Following incubation for 24 h, cells were lysed, and phosphorylation of proteins was estimated by immunoblotting. (E) GP2-293 cells were transfected with expression plasmids of PP2A, and incubated for 24 h. Cells were stimulated with TNF-α for 5 min, and phosphorylation of proteins was analysed by immunoblotting.

Distinct B subunits regulate NF-κB signalling

Together with plasmids encoding Aα, Cα and each B subunit, then the effects of PP2A on IKKβ-EE-mediated phosphorylation of IκBαRR and RelA were investigated by immunoblotting. Expression of the AC core enzyme suppressed phosphorylation of IκBαRR and RelA in a phosphatase activity-dependent manner (Fig. 3C). AC, ABC and AB′′′C reduced IκBα phosphorylation efficiently, whereas AB′C and AB′C had little effect on dephosphorylation of IκBαRR (Fig. 3D). In turn, AC and AB′′′C dephosphorylated RelA efficiently, whereas ABC, AB′C and AB′C had little effect on dephosphorylation of RelA. These results suggest that AC, ABC and AB′′′C attenuate NF-κB activity, at least in part, by dephosphorylation of IκBα.

Then, we investigated the effects of distinct B subunits on phosphorylation of endogenous NF-κB signalling proteins in TNF-α-stimulated cells. Cells were transfected with expression plasmids of PP2A, and then stimulated with TNF-α. AC and AB′′′C suppressed TNF-α-induced phosphorylation of IKKβ and RelA, whereas ABC, AB′C and AB′C had little effect (Fig. 3E). AC, ABC and AB′C reduced TNF-α-induced IκBα phosphorylation, whereas AB′C and AB′C had little effect. These results suggest that functional features of B subunits have physiological significance in NF-κB signalling.

PP2A core enzyme and holoenzyme effectively dephosphorylate IKKβ, IκBα and RelA in vitro

Dephosphorylation of IKKβ, IκBαRR and RelA was analysed by using purified PP2A enzyme in vitro. Expression plasmids encoding Flag-tagged Aα, HA-tagged Cα and HA-tagged B subunits were transfected in cells, and PP2A complexes of various isoforms of B subunit family were purified from cells by using Flag-agarose beads (Fig. 4A). Phosphorylated IKKβ was purified from cells transfected with Flag-IKKβ expression plasmid (Fig. 4B). Following incubation of phosphorylated IKKβ with various purified PP2A core enzyme and holoenzyme, dephosphorylation of IKKβ was analysed by immunoblotting. This assay revealed that purified PP2A complexes of different B subunits effectively dephosphorylate IKKβ, and these complexes showed similar levels of activity (Fig. 4C). Then, we purified phosphorylated complexes of IκBαRR and RelA from cells transfected with plasmids of Flag-RelA, HA-IκBαRR and IKKβEE by using Flag-agarose beads (Fig. 4D). Following incubation of phosphorylated IκBαRR and RelA protein complexes with various purified PP2A core enzyme and holoenzyme, dephosphorylation of IκBαRR and RelA was analysed by immunoblotting. Purified PP2A complexes of different B subunits effectively dephosphorylate IκBαRR and RelA (Fig. 4E). These results indicated that, although all PP2A complexes have an ability to dephosphorylate IKKβ, IκBα and RelA in vitro, AC and AB′′′C preferentially dephosphorylate IKKβ and RelA, and AC, ABC and AB′′′C preferentially dephosphorylate IκBα in vivo, suggesting that intracellular mechanisms may relate to the preference of AC, ABC and AB′′′C to dephosphorylate IKKβ, IκBα and RelA.

Distinct B subunits regulate the binding of PP2A to the substrate proteins in vivo

To resolve the discrepancies in the dephosphorylation of IKKβ and RelA between in vitro and in vivo, we investigated intracellular mechanisms of the preference of AC, ABC, AB′′′C and AB′′′C to dephosphorylate NF-κB signalling proteins. The effects of B subunits on the interaction between PP2A and IKKβ were analysed by immunoprecipitation assay. Cells were transfected with plasmids encoding Flag-tagged CαD85N, HA-tagged Aα, HA-tagged B subunits and HA-tagged IKKβ. PP2A complexes were immunoprecipitated by using Flag-agarose beads from cells, and the association of IKKβ proteins to the PP2A complexes was analysed by immunoblotting (Fig. 5). This assay revealed that IKKβ associates to PP2A, and B′ and B″ subunits block the association. These results suggest that the preference of AC, ABC, AB′′′C and AB′′′C to dephosphorylate NF-κB signalling proteins is regulated by the binding features of B subunits to the substrate proteins in cells.
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A

B

C

D

E

Incubation of Flag-Phospho-IKKβ with purified PP2A complex

Incubation of Flag-Phospho-RelA and HA-IκBαRR complex with purified PP2A
Discussion

Many studies have revealed important and complicated roles of PP2A in IKKβ regulation. For example, it has been reported that PP2A suppresses IKKβ activity in vitro and in vivo [7,15,16]. However, another contradictory study shows that PP2A forms a stable complex with IKKβ and promotes its kinase activity in vivo [28]. We found here that AC and AB″/C dephosphorylate IKKβ in cells, whereas ABC, AB′C and AB′″C do not. These results suggest the possibility that ABC, AB′C and AB′″C complexes positively regulate IKKβ by competing against AC and AB″/C complexes. The data in this study may help to reconcile the seemingly contradictory observations that PP2A both downregulates and promotes IKKβ activity. To confirm the specific regulation by distinct PP2A complexes, we tried to knockdown of multiple isoforms of each B family protein by RNAi. However, RNAi screen in GP2-293 cells did not clarify distinct functions of each isoform of B family protein, because expression levels of B and B‴ family proteins are very low in GP2-293 cells, and these cells express multiple isoforms of B0 and B″ family proteins.

NF-κB signalling is not uniformly regulated by PP2A but rather is subjected to specific regulation by distinct PP2A complexes at multiple steps. In contrast to AC and AB″/C, which dephosphorylate IKKβ, IκBα and RelA, ABC dephosphorylates IκBα but not IKKβ and RelA in cells. These results indicate that ABC suppresses NF-κB activity without inhibition of IKKβ. A recent study revealed that IKKβ not only...
activates NF-κB through the phosphorylation of IkBα but also regulates many cellular functions by phosphorylating various proteins in an NF-κB-independent manner [29]. For example, IKKβ regulates autophagy [30], mRNA stability [31], apoptosis [32], angiogenesis [33] and cellular vesicle trafficking [34] by phosphorylating various proteins that are unrelated to IkBα. Thus, ABC potentially suppresses NF-κB without inhibiting these NF-κB-independent functions of IKKβ, thereby ensuring that PP2A regulates NF-κB activity and IKKβ-mediated cellular responses independently.

Among the four classes of B subunit families, the B′′/Strn family proteins only facilitate dephosphorylation of three proteins including IKKβ, IkBα and RelA. Members of the B′′/Strn family are evolutionarily conserved and have critical roles in biological processes such as development and cell growth [35,36]. B′′/Strn family proteins form a large complex termed Striation-interacting phosphatase and kinase (STRIPAK) along with the germinal centre kinase family and many adaptor proteins, and recruit C subunits via A subunits of PP2A in the complexes. STRIPAK complexes have a critical role in protein dephosphorylation and act as important regulators of multiple vital signalling pathways, including the Hippo pathway, mitogen-activated protein kinases and cytoskeleton remodelling. Recent studies suggest that the dysregulation of STRIPAK complexes correlates with human diseases including cancer [35–37]. Regulation of NF-κB signalling pathway by AB′′C may, therefore, be involved in STRIPAK complex-related biological processes and cancer.

PP2A is a confirmed tumour suppressor protein that is genetically altered or functionally inactivated in many cancers [22–24]. Its A and C subunits are reportedly inactivated through several mechanisms including somatic mutation, phosphorylation, methylation, and/or increased expression of PP2A inhibitors, and inactivation of PP2A is linked to cancer progression. Expression of Bα, Bβ and Bγ is also decreased in many cancers owing to deletion, point mutation and DNA hypermethylation [38–44]. Furthermore, it has been revealed that IKKβ and NF-κB signalling pathways are linked to inflammation and cancer [45]. Therefore, the regulation of IKKβ and NF-κB signalling by distinct PP2A complexes may be involved in multiple human diseases including cancer, and thus might serve as specific and novel targets for disease therapy.

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Author contributions

HK and YT designed and performed the experiments, interpreted the study, and wrote the paper. KO, MK, YN, ET and TH performed the experiments.

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