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Interleukin-32δ interacts with IL-32β and inhibits IL-32β-mediated IL-10 production

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ABSTRACT

There is growing evidence for multifunctional properties of IL-32. We previously demonstrated that IL-32β upregulates IL-10 production through the association with PKCδ. In this study, we examined the effects of other IL-32 isoforms on IL-10 production. We found that IL-32δ decreased IL-10 production and investigated the inhibitory mechanism of IL-32δ. We showed that IL-32δ suppressed IL-32β binding to PKCδ by interacting with IL-32β. The inhibitory effect of IL-32δ on IL-32β association with PKCδ was further verified by immuno-fluorescence staining. The co-localization of IL-32β and PKCδ around the nuclear membrane was disrupted by IL-32δ. Our data therefore indicate that IL-32δ plays an inhibitory role against IL-32β function, which also suggests that IL-32 may be regulated by its own isoform.

Structured summary of protein interactions:
PKC delta physically interacts with **IL-32 beta** by anti bait coimmunoprecipitation (View interaction)
IL-32 beta physically interacts with **PKC delta** by anti tag coimmunoprecipitation (View interaction)
IL-32 beta physically interacts with **IL-32 delta** by anti tag coimmunoprecipitation (1, 2)
PKC delta and **IL-32 beta** colocalize by fluorescence microscopy (view interaction)

1. Introduction

Interleukin-32 (IL-32) was originally reported as a transcript that induced in IL-2-activated natural killer (NK) cell or mitogen-activated T cell [1]. A variety of cell types such as epithelial, endothelial, NK cell, T cell, and dendritic cell express IL-32 [2–4] and the expression of IL-32 is generally induced under inflammation or infection with mycobacterium tuberculosis, Epstein–Barr virus, HIV and Influenza A virus [5–8].

A sequence analysis revealed that IL-32 does not belong to any established cytokine family and that various isoforms exist, but the biological role of each isoform remains to be defined [9]. Although IL-32 has been shown to be secreted when overexpressed [10], multiple studies have demonstrated the preponderance of IL-32 protein in cellular lysates rather than in the supernatant [4,11–13]. Moreover, the cell surface receptor for IL-32 has not yet been identified despite extensive investigation, which raises a question regarding whether IL-32 functions intracellularly or extracellularly. IL-32 has pleiotropic effects on cellular function such as the induction of pro- or anti-inflammatory cytokine [14–16], cell apoptosis [4,17], and cell differentiation [18–20]. Moreover, IL-32 is expressed in various cell types. These evidences suggest the cell-type specific expressions of IL-32 isoforms and their different roles in different cells. Recently, IL-32α is reported to associate with PKCε and STAT3 [21] or with integrins and focal adhesion kinase 1 (FAK1) [22]. IL-32β is also known to promote IL-10 production in a PKCδ-mediated way [23]. In this study, we investigated the effect of IL-32δ and demonstrated how it functions in cells.

2. Materials and methods

2.1. Reagents and cell culture

Human histocytic U937 leukemia cells were grown in RPMI 1640 (WelGENE, Daegu, Korea) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Hyclone, Logan, UT). Human embryonic kidney 293 cells and human lung carcinoma A549 cells were maintained

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in DMEM (WelGENE) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (Hyclone). Phorbol 12-myristate 13-acetate (PMA) was purchased from Sigma (St. Louis, MO), and the PKCδ inhibitor rottlerin was purchased from Calbiochem (San Diego, CA).

2.2. Cloning of IL-32 isoforms

The cDNAs of IL-32 (α, β, and γ) were kindly provided by Dr. SH Kim (Seoul, Konkuk University). IL-32ε cDNA was cloned from THP-1 cells by RT-PCR. IL-32δ and IL-32ζ cDNAs were prepared by PCR using an IL-32δ-specific or IL-32ζ-specific forward primer and IL-32β as the template. The primers are; IL-32δ sense: 5′-GACGAATTCATGAAGAAGCTGAAG-3′, IL-32ζ sense: 5′-GGTGAA TTCATGCAAAATGCAGAA-3′, and common antisense: 5′-GCGCTC GAGTTTTGAGGATT GGGG-3′. Each IL-32 isoform was excised by *Eco*RI and *Xho*I, and then subcloned into pcDNA3.1+ 6×myc vector. IL-32β and IL-32δ were also subcloned into pcDNA3.1+ 5×flag vector.

2.3. Electroporation and ELISA

U937 promonocytic cells were transfected with each IL-32 isoform, or increasing amounts of pcDNA3.1+ 5×flag-IL-32δ by using a Neon™ transfection system (Invitrogen, Carlsbad, CA). The transfected cells were incubated overnight, and 10 nM PMA was then applied for 24 h, after which the culture medium was collected. ELISAs for IL-10, IL-8, and TNF-α were performed using the respective commercial kits (R&D systems, Minneapolis, MN) according to the manufacturer's manuals. For co-localization analyses, A549 lung carcinoma cells were co-transfected with 1 μg of each pcDNA3.1+ 6×myc-IL-32β and pcDNA3.1+ 5×flag-PKCδ, or 1 μg of each plasmid plus 3 μg of pcDNA3.1+ IL-32δ by using a Neon™ transfection system. After overnight incubation, cells were treated with 5 μM of rottlerin for inhibitor-treated sample for 1 h, then with 10 nM PMA for 1 h.

2.4. Immunoprecipitation and Western blotting

HEK293 cells were co-transfected with pcDNA3.1+ 6×myc-IL-32β and pcDNA3.1+ 5×flag-IL-32δ, or pcDNA3.1+ 6×myc-IL-32δ and pcDNA3.1+ 5×flag-IL-32β. To examine IL-32δ effect, HEK293 cells were co-transfected with pcDNA3.1+ 6×myc-IL-32β and pcDNA3.1+ 5×flag-IL-32δ, -PKCδ. Cells were pre-treated for 1 h with the PKCδ inhibitor rottlerin (10 μM), and then treated with PMA (20 nM) for 3 h. HEK293 cells were also co-transfeced with pcDNA3.1+ 6×myc-IL-32β, pcDNA3.1+ 5×flag-PKCδ, and dose-increasing pcDNA3.1+ 5 × flag-IL-32δ. Overnight incubation after transfection, cells were treated with 20 nM PMA for 3 h, then lysed in 50 mM HEPES, pH 7.5, 150 mM NaCl, 5% glycerol, 20 mM β-glycerophosphate, 0.5% NP-40, 0.1% TX-100, and 1 mM EDTA. For immunoprecipitation, cell lysates were mixed with 1 μg of myc antibody, 1 μg of flag antibody, or 3 μg of PKCδ antibody and then pulled down using 35 μl of protein G agarose beads

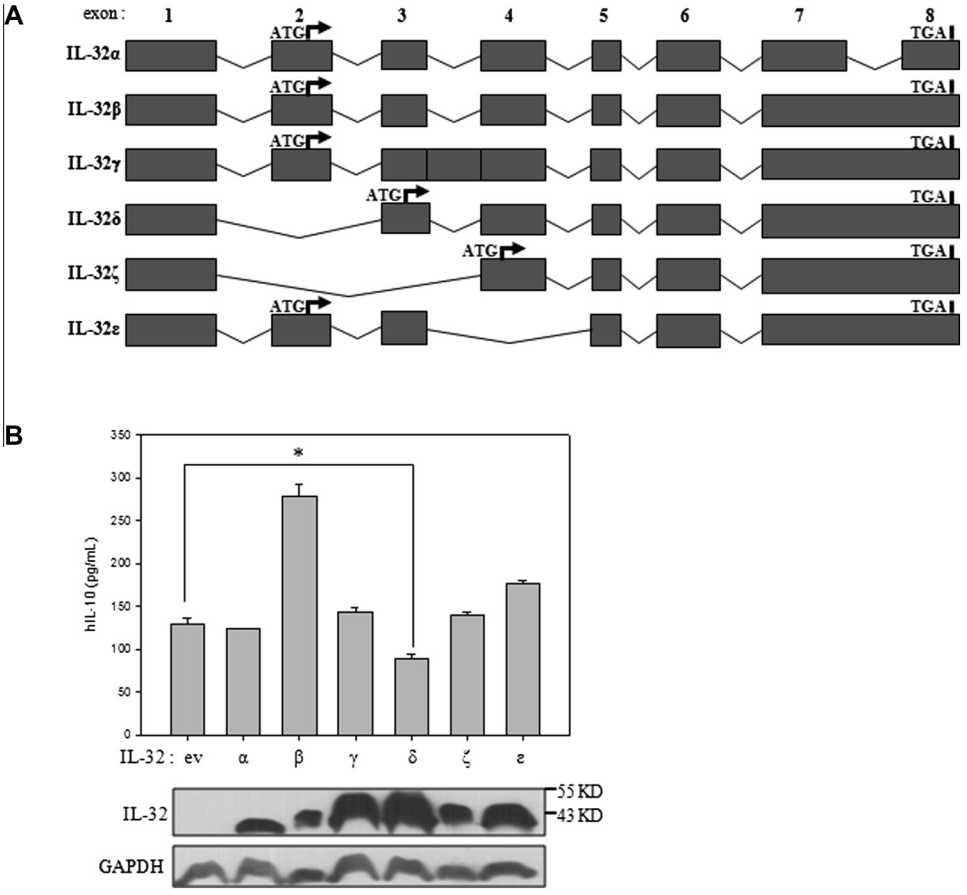

Fig. 1. The effect of IL-32 isoforms on IL-10 production. IL-32 isoforms structures are schematically illustrated. The exon numbers are shown upper the boxes. The schema is based on the literature of Goda et al. [4]. (A). U937 cells were electroporated with each of 3 μg of pcDNA3.1+ 6×myc-IL-32α, β, γ, δ, ζ, ε, or empty vector (ev). Overnight incubation after electroporation, cells were treated with 10 nM PMA for 24 h, then culture media were collected for IL-10 ELISA, and cells were lysed for Western blotting with myc antibody. The expression level of each isoform was confirmed with 20 μg of whole cell lysates. GAPDH was used as a loading control. Values indicate the mean ± S.E.M.; $n = 3$; $^{*}P < 0.05$ for ev vs IL-32δ (B).

(KPL, Gaithersburg, MD). Western blotting was performed using a myc tag antibody (Millipore-Upstate, Bedford, MA) and flag antibody (Sigma).

2.5. Immunofluorescence analyses

For co-localization analyses, A549 cells were grown on covelslip after co-transfection with 6×myc tagged-IL-32β and 5×flag tagged-PKCδ by electroporation. Cells were fixed with 4% paraformaldehyde and permeabilized with 0.5% saponin for 5 min. After blocking with 1% BSA, cells were incubated with primary antibodies in 0.1% saponin. The antibodies were anti-myc (1:200) and anti-flag (1:200). After washing 3 times with PBS, cells were stained with FITC-conjugated anti-mouse (1:400) and rhodamine-conjugated anti-rabbit (1:400) in 0.1% saponine. DAPI was used for nuclei staining. Fluorescence images were obtained by using Olympus BX61-32FDIC upright fluorescence microscope with 100× objective.

2.6. Statistical analysis

Statistical analysis was done by using the unpaired two-tailed Student's *t*-test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. IL-32δ suppresses IL-10 production

Several isoforms of IL-32 are known to produce IL-10. We previously demonstrated that IL-32β upregulates IL-10 production in a PKCδ-mediated way in myeloid cells. We investigated whether there is any other IL-32 isoform to regulate IL-10 production, and found that IL-32δ decreased IL-10 production (Fig. 1B). The gene structure of IL-32 isoforms in this study was schematically depicted (Fig. 1A). We further confirmed the inhibitory effect of IL-32δ on IL-10 production by transfection of IL-32δ in a dose-increasing way into U937 cells, which induced gradual decrease of IL-10 production (Fig. 2A), but in contrast, IL-32δ did not affect the levels of TNF-α and IL-8 (Fig. 2B and C).

3.2. IL-32δ interacts with IL-32β

The down-regulation of IL-10 production by IL-32δ in U937 cells is very interesting because U937 cells abundantly express

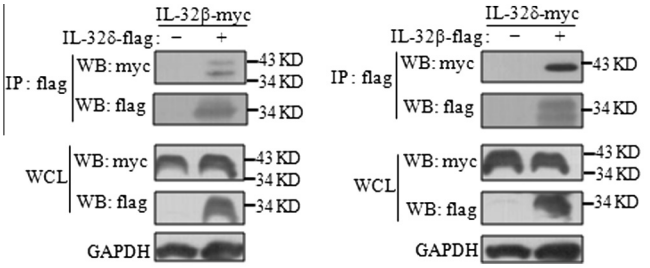

Fig. 3. IL-32δ interacts with IL-32β. HEK293 cells were co-transfected with pcDNA3.1+ 6×myc-IL-32β and pcDNA3.1+ 5×flag-IL-32δ (A), or pcDNA3.1+ 6×myc-IL-32δ and pcDNA3.1+ 5×flag-IL-32β (B). After overnight incubation, immunoprecipitations were performed with 1 μg of flag antibody, then Western blotting were carried out with the both antibodies. The expression levels of the transfected genes were confirmed with 30 μg of whole cell lysates (WCL). Protein size marker is indicated at right of each box.

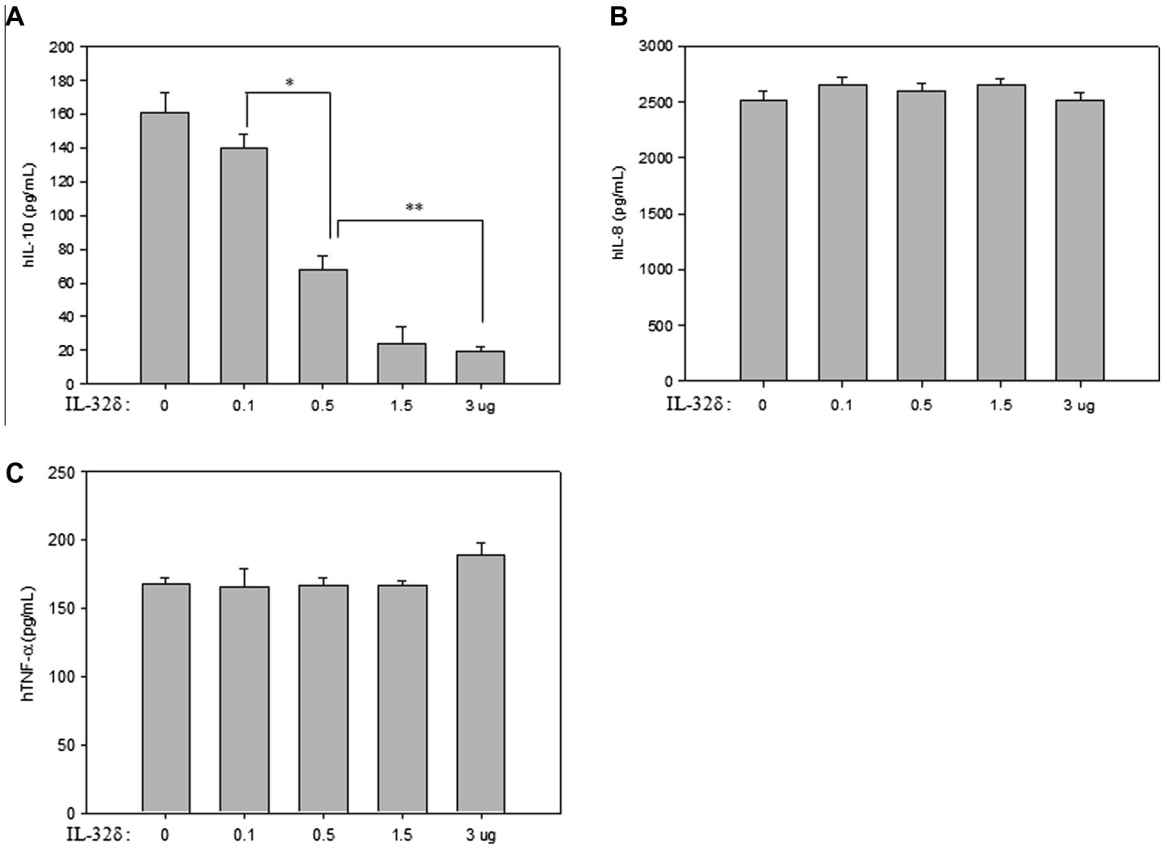

Fig. 2. IL-32δ inhibits IL-10 production in U937 cells. U937 cells were electroporated with increasing amounts of pcDNA3.1+ 5×flag-IL-32δ (0, 0.1, 0.5, 1.5, and 3 μg). The total amount of the introduced DNA was adjusted to 3 μg with an empty vector. After overnight incubation, the cells were treated with 10 nM PMA for 24 h and the culture media were then collected for ELISA for IL-10 (A), IL-8 (B), and TNF-α (C). Values indicate the mean ± S.E.M.; $n = 4$; $^{*}P < 0.03$ for IL-32δ 0.1 vs 0.5 μg, $^{**}P < 0.05$ for IL-32δ 0.5 vs 3 μg.

endogenous IL-32β and IL-32β is involved in IL-10 production. The promonocytic leukemia U937 cell line is useful for studying IL-32 function because it expresses the transcripts of IL-32β, IL-32γ, IL-32ε, and IL-32η (data not shown). However, only IL-32β has been characterized with regard to the upregulation of IL-10 in U937 cells [16]. We previously showed that IL-32β associated with PKCδ, which induced IL-10 upregulation [23]. Hence, we examined whether IL-32δ has any effect on IL-32β function. We found that IL-32δ interacted with IL-32β (Fig. 3), which suggests that IL-32δ is involved in IL-32β function.

3.3. IL-32δ inhibits the association of IL-32β with PKCδ

We examined the interaction profile between IL-32β, IL-32δ, and PKCδ by using immunoprecipitation assays after cotransfection of HEK293 cells with the genes coding for these proteins. To make the results clear, we transfected HEK293 cells because this cell line does not express any endogenous IL-32 isoform (data not shown). As shown in Fig. 4A, IL-32β associated with IL-32δ regardless of PMA treatment (lane 1). However, the interaction of IL-32β with PKCδ was PMA-dependent (lane 1 vs lane 2) as confirmed earlier [23]. Notably, when comparing lane 2 with lane 4, IL-32δ co-expression with IL-32β and PKCδ resulted in the decrease of the association between IL-32β and PKCδ, which means that IL-32δ inhibited the interaction of IL-32β and PKCδ. A PKCδ-specific inhibitor, rottlerin, did not inhibit the interaction of IL-32δ with IL-32β (lane 3). This result means that PKCδ did not affect the interaction of IL-32δ and IL-32β (lane 6). The same results were obtained in an immunoprecipitation experiment with a PKCδ antibody (Fig. 4B), where IL-32δ suppressed the association of IL-32β and PKCδ (lane 2 vs lane 3). As consistent with Fig. 4A, IL-32β interacted with PKCδ upon PMA stimulation (lane 1 vs lane 2).

3.4. IL-32δ negatively regulate IL-32β function through the dynamic interaction with each other

To further verify that IL-32δ is a negative regulator of IL-32β on the IL-10 production, we next investigated the dynamics by cotransfection with IL-32β, PKCδ, and increasing amounts of IL-32δ. When PKCδ was immunoprecipitated, the associated IL-32β gradually decreased as the amount of transfected IL-32δ increased. In the immunoprecipitation of IL-32β, the pulled-down PKCδ decreased, whereas the co-immunoprecipitated IL-32δ increased (Fig. 4C and D). These results confirm that the association of IL-32β with PKCδ is suppressed by IL-32δ.

We next verified the association profile of IL-32δ, IL-32β, and PKCδ by immunofluorescence staining in A549 cells. Both IL-32β and PKCδ were distributed in the cytosol in normal condition. However, they co-localized around the nucleus upon PMA treatment. Rottlerin disrupted their co-localization. As consistent with the immunoprecipitation data, IL-32δ induced the dissociation of IL-32β from PKCδ (Fig. 5). These data confirm the inhibitory effect of IL-32δ on IL-32β function.

4. Discussion

IL-32 is widely known as a proinflammatory cytokine whose biological effects have been extensively studied. It is implicated

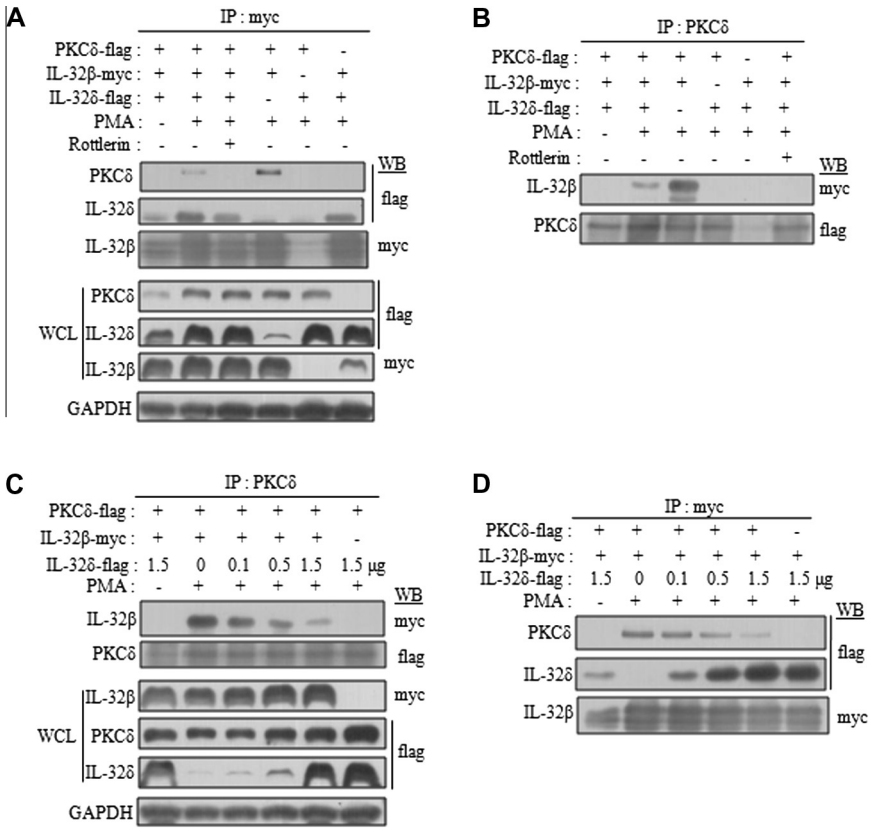

Fig. 4. The inhibitory effect of IL-32δ on the interaction of IL-32β and PKCδ. HEK293 cells were cotransfected with 6×myc-tagged IL-32β, 5×flag-tagged PKCδ, and 5×flag-tagged IL-32δ (A). After overnight incubation, a PKCδ specific inhibitor rottlerin 5 μM was treated for 1 h for inhibitor-treated samples, and then 20 nM PMA was treated for 3 h. The immunoprecipitation assays were performed by using 1 μg of myc antibody (A) or 3 μg of PKCδ antibody (B). HEK293 cells were co-transfected with IL-32β, PKCδ, and increasing amounts of IL-32δ (0, 0.1, 0.5, and 1.5 μg). The total amount of DNA for transfection was adjusted to 1.5 μg by using an empty vector. Immunoprecipitation was performed with 3 μg of the PKCδ antibody (C) or 1 μg of the myc antibody (D). The expression levels of the transfected genes were determined by Western blottings with 30 μg of whole cell lysates (WCL).

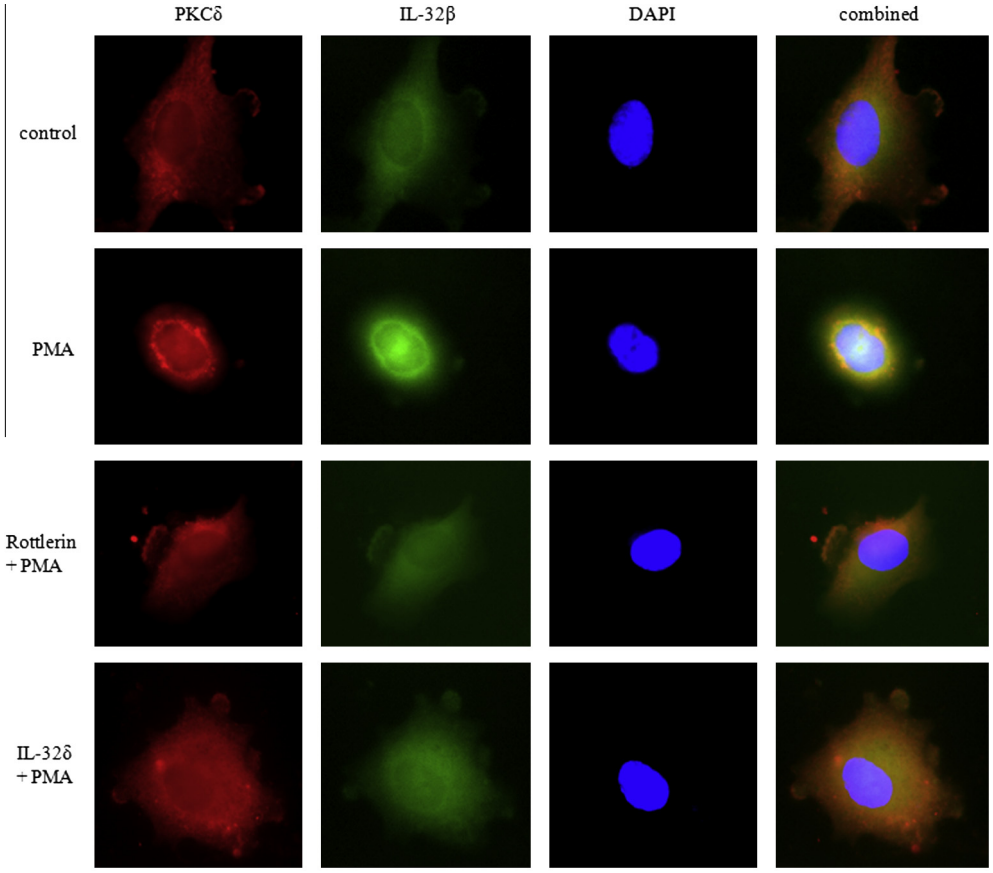

Fig. 5. IL-32δ disrupts the association of IL-32β and PKCδ. A549 lung carcinoma cells were electroporated with 1 μg of the expression plasmids for pcDNA3.1+ 6×myc-IL-32β, pcDNA3.1+ 5×flag-PKCδ, and 3 μg of pcDNA3.1+ IL-32δ. After overnight incubation, cells were treated with 20 nM of PMA for 3 h. Rottlerin (5 μM) was treated 1 h before PMA treatment. Immunofluorescence signals were detected for PKCδ (red), IL-32β (green), and the combined (yellow) by using upright fluorescence microscope.

in inflammatory diseases such as rheumatoid arthritis [8,24,25], ulcerative colitis, Crohn's disease [13,26], and psoriasis [2,27,8] as well as obstructive pulmonary disease [27], viral infections, bacterial infection, and cancer [29,30]. IL-32α and IL-32β have been studied for their proinflammatory effects. IL-32β is known to involve in activation-induced cell death in T cells [4]. IL-32γ is reported to be the most active isoform [31] and that induces the differentiation of blood monocytes into macrophage-like cells [20] and CD14+ monocytes into osteoclasts [18], as well as the maturation and activation of dendritic cell [32]. However, IL-32γ tends to be spliced into shorter isoforms when ectopically expressed in cells. Splice-resistant IL-32γ mutant shows much higher induction of proinflammatory cytokines than IL-32β [15]. IL-32β and IL-32γ are also known for their effect on IL-10 induction [16,33]. However, such pleiotropic effects have only been reported for several isoforms thus far, even though more than 9 isoforms are found in the GenBank Database.

Recently, IL-32 has been reported to interact with the extracellular domain of integrins and intracellular proteins such as paxillin and focal adhesion kinase-1 (FAK1) through its alpha helix region. IL-32β is the most abundantly and broadly expressed isoform and is known to promote IL-10 production in U937 cells upon PMA stimulation as well as in the purified monocyte-derived dendritic cells by LPS stimulation [16].

Many proteins have their alternatively spliced variants. The existence of multiple isoforms of a protein owing to alternative splicing may provide it functional relatedness. However, it is less common that alternatively spliced isoforms of a protein interact with each other to regulate its own function. One such a case is a putative tumor suppressor protein Fbw 7, which is a specificity factor for the Skp 1-Cul 1-F-box protein ubiquitin ligase complex. Fbw 7 has three alternative splice variants that are unique only at their N-termini but are otherwise identical as with IL-32 protein. Fbw 7 promotes cyclin E turnover efficiency through its isoform interaction [34]. In this study, we investigated the effects of other isoforms of IL-32 on IL-10 production. IL-32δ transcript is expressed in T cells and NK cells [4,10], but its function remains to be elucidated. We showed for the first time that IL-32δ interaction with IL-32β suppressed the association of IL-32β with PMA-activated PKCδ, resulting in the decrease of IL-10 production in U937 cells. The results in Fig. 4C and D suggest that IL-32δ competitively inhibit the binding of IL-32β to PMA-activated PKCδ. Our data imply that there may be more interacting pairs between IL-32 isoforms and there may be more functional relationship between IL-32 isoforms.

Acknowledgments

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