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Short communication

Interaction network mapping among IL-32 isoforms

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ABSTRACT

IL-32 has been studied for its pleiotropic effects ranging from host immune responses to cell differentiation. Although several IL-32 isoforms have been characterized for their effects on cells, the roles of the others remain unclear. We previously reported that IL-32δ interacted with IL-32β and inhibited IL-32β-mediated IL-10 production. Thus, we performed comprehensive analyses to reveal more interactions between IL-32 isoforms in this study. We screened the interactions of 81 combinations of nine IL-32 isoforms by using a yeast two-hybrid assay, which identified 13 heterodimeric interactions. We verified these results by using reciprocal immunoprecipitation assays and reconfirmed 10 interactions, and presented the interaction network map between IL-32 isoforms. Our data suggest that IL-32 may have diverse intracellular effects through the interactions with its different isoforms.

1. Introduction

IL-32 is a proinflammatory cytokine whose biological effects have been extensively studied. IL-32 is known to be a multifunctional cytokine that inducing inflammatory responses [1,2], apoptosis [3,4], and differentiation [5,6]. Although an expressed sequence tag (EST) clone of equine, bovine, ovine, and swine IL-32 is found in the NCBI databank, IL-32 is expressed only in primates, which implies that IL-32 may be a human/primate specific gene [2,7]. The gene encoding IL-32 is located on human chromosome 16p13.3 and consists of 8 exons, but the first exon is not translated into protein [2,8]. A variety of types of cell such as epithelial cells, NK cells, T cells, dendritic cells, and endothelial cells express IL-32 [9,10], which suggest the cell-type specific expressions of IL-32 isoforms and their different roles in different cells [5,6,9,11].

There are more than 9 isoforms of IL-32 in the GenBank Database, all of which are generated by alternative splicing. However, multifunctional effects of IL-32 have only been reported for several isoforms thus far. Recently IL-32α is reported to associate with PKCε and STAT3 [12] or with integrins and focal adhesion kinase 1 (FAK1) [13]. IL-32β interacts with PKCδ and C/EBPα, which results in IL-10 upregulation [14]. This evidence suggests that IL-32 plays an intracellular mediatory role through the interaction with signaling molecules. Moreover, we recently reported that IL-32δ interacts with IL-32β [15]. The interaction of IL-32δ with IL-32β inhibits IL-32β function of IL-10 production, and this is the first report of pairing between IL-32 isoforms, and also suggest that there may be more interactions between IL-32 isoforms.

In the present study, we investigated the molecular interactions among IL-32 isoforms with the expectation that the functional diversity of IL-32 would be explained by the various interactions among the isoforms.

2. Materials and methods

2.1. Reagents and cell culture

HEK293 cells were grown in Dubellco's modified eagle's medium (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). Yeast strain AH109 was kindly provided by Dr. JH An (Seoul, Konkuk University). Phorbol 12-myristate 13-acetate (PMA) and 3-amino-1,2,4-triazole (3-AT) were purchased from Sigma (St. Louis, MO).

Abbreviations: PMA, phorbol 12-myristate 13-acetate; LPS, lipopolysaccharide; 3-AT, 3-amino-1,2,4-triazole.

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2.2. 5′ Rapid amplification of cDNA ends (5′ RACE)

This experiment was performed using a FirstChoice® RLM-RACE Kit (Ambion, St. Austin, TX) according to the manufacturer's instructions. Briefly, total RNA was extracted from monocyte-derived dendritic cells that had been purified from human peripheral blood mononuclear cells (PBMCs) after stimulation with 1 μg/ml of lipopolysaccharide (LPS) as described previously [16]. Ten micrograms of the total RNA were dephosphorylated at the 5′ end by calf alkaline phosphatase, decapped by tobacco acid pyrophosphatase, ligated with a 5′ RACE adapter by T4 RNA ligase, and then reverse transcribed. The PCR-amplified products were separated on 1% agarose gel. Bands were extracted from the gel and cloned into a T&A cloning vector (Real Biotech Corporation, Taiwan). Every PCR product was analyzed by sequencing.

2.3. Cloning of IL-32 isoforms and yeast two-hybrid assay

The cDNAs of IL-32 (α, β, and γ) were kindly provided by Dr. SH Kim (Seoul, Konkuk University). IL-32ε and IL-32η cDNAs were cloned from THP-1 and U937 promonocytic cells by RT-PCR, respectively. 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′-GGT GAATTCATGCAAAATGCAGAA-3′, sense primer for other IL-32 isoforms : 5′-GCTGAATTCATGTGCTTCCCGAAG-3′, and common antisense : 5′-GCGCTCGAGTTTTGAGGATTGGGG-3′. IL-32θ and IL-32s were cloned as described above. Each IL-32 isoform was excised by *Eco*RI and *Xho*I, and then subcloned into the GAL4 activation domain vector (pGAD T7) at *Eco*RI and *Xho*I sites or the GAL4 DNA binding domain vector (pGBK T7) at the *Eco*RI and *Sal*I sites. Interactions among the isoforms were determined according to the interaction-dependent induction of the reporter genes *HIS3* and *lacZ*. The GAL4 activation domain fusion protein and GAL4 DNA binding domain fusion protein were coexpressed in the yeast strain AH109. Interactions in the two-hybrid assay were detected by cell growth on histidine-depleted synthetic yeast medium containing 3-amino-1,2,4-triazole ($Leu^-/Trp^-/His^-/Ade^-$/3.5 mM, 3-AT), which is a competitive inhibitor of yeast *HIS3*. The pGBK T7 and pGAD T7 vectors were kindly provided by Dr. JH An.

2.4. Immunoprecipitation and western blotting

Nine IL-32 isoforms were subcloned into pcDNA3.1 + 6 × myc and pcDNA3.1 + 5 × flag vectors. Immunoprecipitation was performed reciprocally according to the results of the yeast two-hybrid assay. Overnight incubation after co-transfection, HEK293 cells were 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. Western blotting was performed using a myc tag antibody (Millipore-Upstate, Bedford, MA) and flag antibody (Sigma). For immunoprecipitation, cell lysates were mixed with 1 μg of myc or flag antibody, and then pulled down using 35 μl of protein G agarose beads (KPL, Gaithersburg, MD).

3. Results and discussion

3.1. Screening of interactions among IL-32 isoforms by a yeast two-hybrid assay

Recently, we demonstrated the interaction of IL-32δ with IL-32β, which suppressed IL-32β-mediated IL-10 upregulation by inhibiting IL-32β association with PKCδ [15]. Thus, we speculated that there may be more interactions between IL-32 isoforms. IL-32 has at least 9 isoforms including the 2 new isoforms identified in this study by 5′ RACE, which are IL-32θ and IL-32 small (IL-32s). IL-32θ is deficient of exon 6, and IL-32s contains the second half of exon 7 and full exon 8. We deposited the sequences in the GenBank with the accession numbers FJ985780 and FJ985781, respectively. IL-32s is the most shortest isoform of 280 base pairs. We performed a yeast two-hybrid assay in reciprocal way because of the directionality of two-hybrid assay for some protein pairs. Interactions between pairs of IL-32 isoforms were investigated for 81 combinations (9 × 9 isoforms, and 100 combinations in total including the empty vectors) in a yeast two-hybrid assay by monitoring the induction of the *HIS3* gene. Expression of *HIS3* was detected by cell growth on histidine-depleted medium in the presence of 3.5 mM 3-AT, which is a competitive inhibitor of *HIS3*. By using appropriate concentration of 3-AT, false positives can be eliminated. Of the 81 combinations, 13 heterodimeric IL-32 isoform pairs (α–γ, α–ε, β–δ, β–ζ, β–η, γ–δ, γ–ζ, γ–η, γ–θ, δ–ζ, δ–ε, ε–ζ, and ε–θ) demonstrated positive interactions (Fig. 1A). There was no interaction between homologous pairs. Interestingly, pGBK T7-IL-32α and IL-32β showed no interaction with other pGAD T7-IL-32 isoforms. However, this directionality of protein interaction in a two-hybrid is not unusual [17,18]. In this case, it may be because GAL4 DNA binding domain affected the geometry of the interacting motif of IL-32α and IL-32β. IL-32s did not interact with any isoform.

3.2. Interaction network mapping

We verified the interactions between IL-32 isoforms identified in the yeast two-hybrid assay by using reciprocal immunoprecipitation. We subcloned each isoform of IL-32 into pcDNA3.1 + 6 × myc or pcDNA3.1 + 5 × flag vector. After cotransfection of HEK293 cells with the pairs identified in the two-hybrid assay, reciprocal immunoprecipitation was performed using myc antibody or flag antibody. The interaction pairs (β–δ, β–η, and δ–ζ) were reconfirmed, except the pair of IL-32β and IL-32ζ (Fig. 1B and C). IL-32β had a strong interaction with IL-32η in the immunoprecipitation assay although it was a weak interaction in the yeast two-hybrid assay. As shown in Fig. 1D and E, most pairs of IL-32 isoforms (γ–δ, γ–ζ, γ–η, γ–θ, ε–δ, ε–ζ, and ε–θ) that were positive interactions in the yeast two-hybrid assay were reconfirmed by immunoprecipitation. It was unexpected that IL-32α did not immunoprecipitate with IL-32γ or IL-32ε because IL-32α was shown to interact strongly with IL-32γ or IL-32ε in the yeast two-hybrid assay. We also examined whether the non-interacting pairs from a two-hybrid would turn to be positive in immunoprecipitations. We performed immunoprecipitation assays with several negative interaction pairs of IL-32 isoforms (β–α, β–ζ, θ–α, θ–ζ, and θ–η) and demonstrated that those non-interacting pairs from a two-hybrid still showed no interaction in immunoprecipitation assays. The positive interaction pair of IL-32γ and IL-32δ from Fig. 1D was included for comparison (Fig. 1F). We compared the results of the yeast two-hybrid and those of immunoprecipitation assays (Fig. 2A), and generated an interaction network map among IL-32 isoforms (Fig. 2B).

We at first expected that the interactions between IL-32 isoforms might be mediated by the RGD and DDX motifs, which are located in the C-termini and N-termini of IL-32 peptide, respectively. However, the interaction pattern was irrelevant to both the motifs. IL-32α contains a four α-helix bundle structure and IL-32β has an additional short α-helix, which is a typical protein–protein interaction motif [13]. Meanwhile, we observed that each IL-32 isoform was detected as doublets in western blotting, which might be due to the protein modification such as phosphorylation (unpublished data). We speculate that the interaction status between IL-32 isoforms may be affected by the phosphorylation