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Selection of cDNA candidates that induce oligomerization of NLRP3 using a chimeric receptor approach

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Since diverse cellular events are regulated by protein oligomerization, identification of molecules that affect oligomerization of a target protein is important for understanding cellular physiology and developing therapeutics. In this study, we aimed to screen cDNA candidates that induce oligomerization of NLRP3, which is one of the important inflammatory sensor proteins, in mammalian cytoplasm. In our screening method, the chimera composed of NLRP3 and the kinase domain of c-kit, one of the receptor tyrosine kinases (RTKs) activated by oligomerization, is expressed in cytoplasm of an IL-3-dependent mammalian cell line. The cells are then transduced with a cDNA library, and cultured in the absence of IL-3. If the transduced cDNA is a NLRP3 activator, the kinase domain of the NLRP3-c-kit chimera is activated by oligomerization, which induces cell growth even in the absence of IL-3. Using this system, constitutive oligomers of two NLRP3 variants were clearly detected by cell growth. Furthermore, cDNA screening resulted in identification of three distinct cDNAs that are potential candidates of NLRP3 activators. These results demonstrate the utility of our chimeric receptor-based system for screening candidates that induce oligomerization of a target protein.

[**Key words:** Protein oligomer; Chimeric receptor; Tyrosine phosphorylation; cDNA library; Mammalian cell]

Protein oligomerization is important for regulating the activity of proteins in living cells. Growth factor receptors are activated by ligand-induced dimerization, which induces full activation of their kinase domain and triggers subsequent signal transduction cascades (1–3). Dimerization is required to be functional for transcription factors with the motif or domain such as basic helix-loop-helix (bHLH), basic leucine zipper (bZIP), Rel homology, and MADS box, and many transcription factors can form homo- or heterodimers that regulate the specificity of their target DNA and their binding partners (4,5). In Fas-mediated death signaling, the Fas ligand forms a trimer and induces trimerization of Fas, which induces death-inducing signaling complex (DISC) which is composed of Fas-associated death domain (FADD) and procaspase-8, the latter of which is activated by dimerization and oligomerization to initiate downstream caspase cascade reactions (6,7). Apoptotic protease-activating factor-1 (Apaf-1) protein belongs to the AAA^+ family of ATPases and forms heptamers in response to cytochrome c which is released from mitochondria in the intrinsic apoptotic pathway (8,9). Since diverse cellular signaling events are regulated by protein oligomerization, identification of molecules that affect oligomerization of a target protein is important for understanding cellular physiology and developing therapeutics.

Nod-like receptors (NLRs) are intracellularly expressed pattern-recognition receptors that recognize specific microbial components, termed pathogen-associated molecular patterns (PAMPs), and have recently been recognized as important sensor proteins in immune system (10,11). Intriguingly, NLR family, pyrin domain-containing 3 (NLRP3), which is one of the NLR family members, recognizes more diverse PAMPs than the other NLR members, and most importantly, is activated also by non-microbial risk factors, termed damage-associated molecular patterns (DAMPs) (12–18). The NLRP3 is composed of four domains, namely pyrin domain (PYD), domain present in *NAIP*, *CIITA*, *HET-E*, and *TP-1* (NACHT), NACHT-associated domain (NAD), and leucine-rich repeats (LRR) (19). In the proposed activation mechanism, NLRP3 takes a closed conformation by intramolecular domain interaction between LRR and NACHT, which inhibits oligomerization of NLRP3 (20,21). Upon activation, this inactive conformation is released by ligand binding or as-yet-unidentified mechanism, leading to oligomerization of NLRP3 through NACHT–NACHT homo-interactions (Fig. 1A). The NLRP3 oligomers subsequently form the complex called 'inflammasome' (22,23). The PYD of NLRP3 recruits apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC), which subsequently recruits caspase-1, leading to the activation of caspase-1 and digestion of the propeptides of inflammatory cytokines interleukin (IL)-1β and IL-18 (24,25). This cleavage results in secretion of these inflammatory cytokines, and initiates the inflammatory responses. The discovery of structurally diverse activators for NLRP3 has led to the hypothesis that common intracellular factors regulate the activation of NLRP3. Since most of the NLRP3 activators are related to reactive oxygen species (ROS) generation, redox- or mitochondrial damage-related molecules such as thioredoxin-interacting protein (TXNIP) and oxidized mitochondrial DNA are reported as potential ligands for NLRP3

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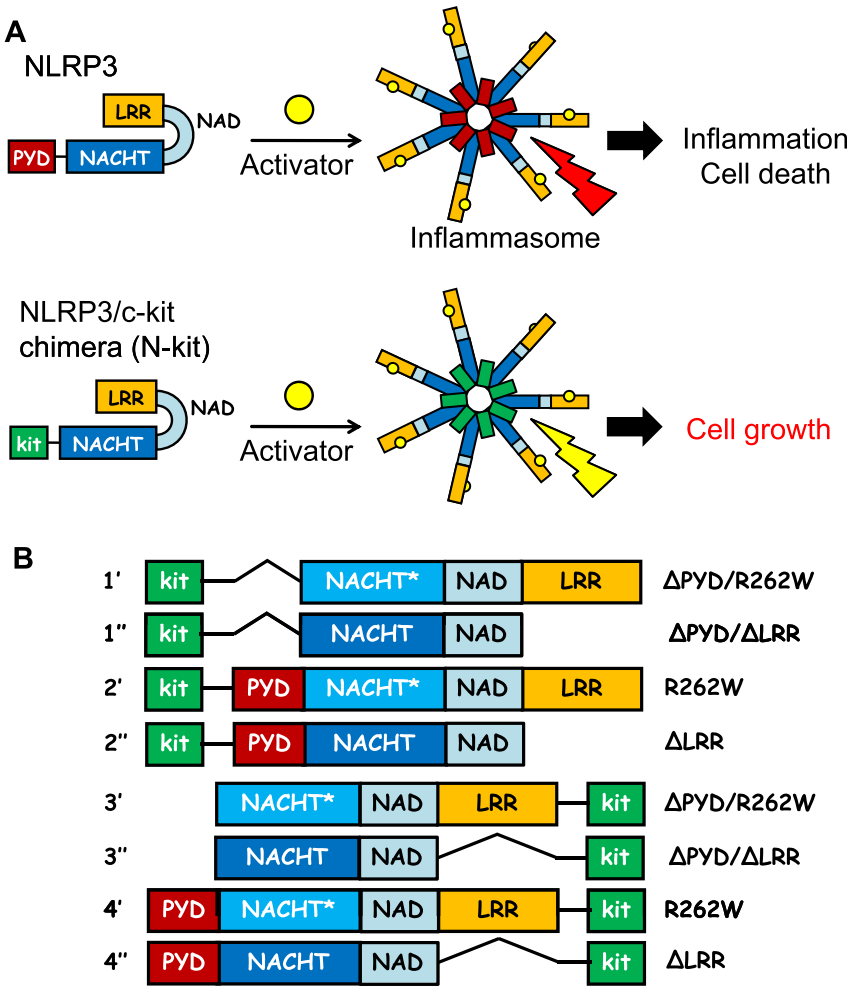

FIG. 1. Design of the NLRP3/c-kit chimeras. (A) Schematic illustration of the activation mechanisms of native NLRP3 and N-kit. NLRP3 takes monomeric inactive conformation in the absence of activators. Once activated, NLRP3 homo-oligomerizes through the NACHT domain, forms the active inflammasome complex through the PYD domain, and triggers inflammatory responses and cell death. In contrast, N-kit is a fusion protein composed of NLRP3 and the growth-signaling domain of c-kit. When activated, N-kit homo-oligomerizes through the NACHT domain and triggers a cell-growth signal. (B) The domain structures of constitutively active N-kit chimeras (caN-kits) constructed in this study. The R262W mutation in the NACHT domain is represented as NACHT*. All constructs encode a Myc tag at the C-terminus.

(26,27). In addition, increasing evidences suggest that NLRP3 is activated through elevated Ca^{2+} levels in cytoplasm, which is closely linked to endoplasmic reticulum stress (28,29). However, the precise mechanism for activation of NLRP3 still remains to be determined (29). Finding other upstream activators would be important to elucidate overall activation mechanism of the NLRP3 inflammasome. Although robust yeast two-hybrid screening has been used for finding direct endogenous binders for NLRP3 (26,30,31), yeast nucleus would be environmentally different from mammalian cytoplasm where the native NLRP3 functions. Furthermore, because upstream activators may not directly bind to NLRP3, binding-based assays cannot entrap all activators in principle.

We aimed to develop a novel platform for screening factors that induce oligomerization of NLRP3 directly in mammalian cytoplasm. Such a platform would enable us to entrap upstream activators as well as direct ligands. Here we focus on c-kit (32–34), one of the receptor tyrosine kinases (RTKs) activated by dimerization or higher-order oligomerization, which is analogous to NLRP3 in terms of the activation mechanism. In our screening method, the chimera composed of NLRP3 and the kinase domain of c-kit is expressed in cytoplasm of an IL-3-dependent mammalian cell line. The cells are then transduced with a cDNA library, and cultured in the absence of IL-3. If the transduced cDNA is a NLRP3 activator, the kinase domain of the NLRP3-c-kit chimera is activated by oligomerization, which would induce cell growth even in the absence of IL-3 (Fig. 1A). As a proof-of-concept experiment, we firstly employed constitutively active NLRP3 mutants to investigate whether the NLRP3-kinase chimera could transduce a growth signal. Next, we performed cDNA library screening in the cells expressing the NLRP3-kinase chimera based on growth induction and subsequent recovery of cDNA sequences by genomic PCR. After cloning and specificity check of the cDNAs, we identified three cDNA candidates that induce activation of NLRP3.

MATERIALS AND METHODS

Plasmid construction The retroviral plasmid pMK-stuffer-myc-IG, which encodes a myc tag and an internal ribosomal entry site (IRES)-enhanced green fluorescent protein (EGFP) cassette, was used as a starting material. First, the undesirable *Sal*I site in pMK-stuffer-myc-IG was deleted by digestion with *Sal*I, blunting and self-ligation to make pMK-stuffer-myc-IG(-SalI). To insert a $(G_4S)_3$ linker sequence, pIT2-31IJ3 encoding anti-fluorescein single-chain Fv (35) was digested with *Sfi*I and *Not*I, and subcloned into pMK-stuffer-myc-IG(-SalI), resulting in pMK-V_H-$(G_4S)_3$-V_L-stuffer-myc-IG. The intracellular domain of c-kit was amplified with PCR using two primers (SfiI-kit primer_F, 5′-GCCG GGGCCCAGCCGGCCACCATGGGTACCATGGGTACCTACAAATATTTACAGAAAC-3′; kit-XhoI primer_R, 5′-GGGCTCGAGACATCGTCGTGCACAAGCAG-3′) and pMK-ΔL-stuffer-ΔTM-kit-Flag-IG (manuscript in preparation) as the template. The