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Proteinase 3-processed form of the recombinant IL-32 separate domain

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Interleukin-32 (IL-32) induces a variety of proinflammatory cytokines and chemokines. The IL-32 transcript was reported originally in activated T cells; subsequently, it was demonstrated to be abundantly expressed in epithelial and endothelial cells upon stimulation with inflammatory cytokines. IL-32 is regulated robustly by other major proinflammatory cytokines, thereby suggesting that IL-32 is crucial to inflammation and immune responses. Recently, an IL-32α-affinity column was employed in order to isolate an IL-32 binding protein, neutrophil proteinase 3 (PR3). Proteinase 3 processes a variety of inflammatory cytokines, including TNFα, IL-1β, IL-8, and IL-32, thereby enhancing their biological activities. In the current study, we designed four PR3-cleaved IL-32 separate domains, identified by potential PR3 cleavage sites in the IL-32α and γ polypeptides. The separate domains of the IL-32 isoforms α and γ were more active than the intrinsic α and γ isoforms. Interestingly, the N-terminal IL-32 isoform γ separate domain evidenced the highest levels of biological activity among the IL-32 separate domains. [BMB reports 2008; 41(11): 814-819]

INTRODUCTION

Interleukin-32 (IL-32) is a new cytokine which was described originally as a transcript (NK4) that is expressed selectively in activated natural killer or T cells (1). Although IL-32 does not share sequence homology with the known inflammatory cytokine families, it exhibits the inflammatory property of inducing other inflammatory cytokines (2). In an attempt to identify the genes induced by IL-18, we discovered a new cytokine, IL-32, (2) which stimulates monocytic cells to generate proinflammatory cytokines including IL-1β, IL-6, TNFα, and chemokines (3). In addition, *in vitro* studies of IL-32 regulation reveal that IL-32 is induced predominantly by the proinflammatory cytokines IL-1β, TNFα, and IFNγ (4).

The IL-32 ligand-affinity column isolated a specific 30-kDa protein, which was determined to be a neutrophil proteinase 3 (PR3) (5). PR3 exists in both soluble and membrane-associated forms and is the principal autoantigen in the systemic vasculitic disease, Wegener's granulomatosis (6, 7). After limited cleavage by PR3, IL-32α evidences enhanced biological activity, as evaluated via mouse macrophage inflammatory protein-2 (MIP-2) and human IL-8 induction (5). The primary function of PR3 involves the degradation of extracellular proteins at inflammation sites; however, excessive proteolytic activity exerts harmful effects.

IL-32 is expressed abundantly in rheumatoid arthritis synovial tissue biopsies, but is not detected in synovial tissues from patients with osteo-arthritis (8). After the administration of human recombinant IL-32 into the knee joints of naïve mice, joint swelling associated with an influx of inflammatory cells and cartilage damage were observed. IL-32-driven joint swelling and cell influx was reduced markedly in TNFα-deficient mice, although the loss of proteoglycan remained unaffected (8). These data indicate that IL-32-induced rheumatoid arthritis is dependent on TNFα.

The mouse model of human IL-32β overexpression via bone marrow transplantation (BM-human IL-32) evidences the increased expression and secretion of TNFα, IL-1β, and IL-6 in spleen cells following lipopolysaccharide stimulation. BM-human IL-32 mice evidence the onset of collagen and antibody-induced arthritis and trinitrobenzene sulfonic acid-induced colitis. In addition, the inhibition of TNFα reduces the inflammatory effects of human IL-32β (9). Moreover, IL-32 induces the release of prostaglandin E2 in mouse macrophages and human blood monocytes, which is a crucial property of inflammatory cytokines (8).

IL-32 synergizes with the nucleotide-binding oligomerization domain (NOD) 1 and 2 specific muropeptides of peptidoglycans for the release of IL-1β and IL-6 (3). The synergistic effect of IL-32 and muramyl dipeptide (MDP) on cytokine production is abrogated in cells collected from patients with Crohn's disease bearing the NOD2 mutation, which induces an insertion of cytidine at position 2030. The synergistic effect of IL-32 on cytokine induction by muropeptides is lacking in macrophages from NOD1-deficient mice (3). IL-32 is induced by *Mycobacterium*

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tuberculosis and *M. bovis* BCG, as well as by either lipopolysaccharide or mycobacteria. Moreover, *M. tuberculosis*-induced IL-32 generation is dependent on endogenous IFNγ (10).

In this study, we have expressed PR3-cleaved recombinant IL-32 separate domains and have characterized the biological activity of the different domains. Four different PR3-cleaved IL-32 separate domains were cloned, and each IL-32 separate domain protein was generated. The biological activity of PR3-cleaved IL-32 separate domains was assessed via multiple bioassays. Interestingly, the N-terminal IL-32γ separate domain evidenced the highest levels of biological activity, inducing a variety of inflammatory cytokines in primary peripheral blood mononuclear cells and human lung epithelial cells.

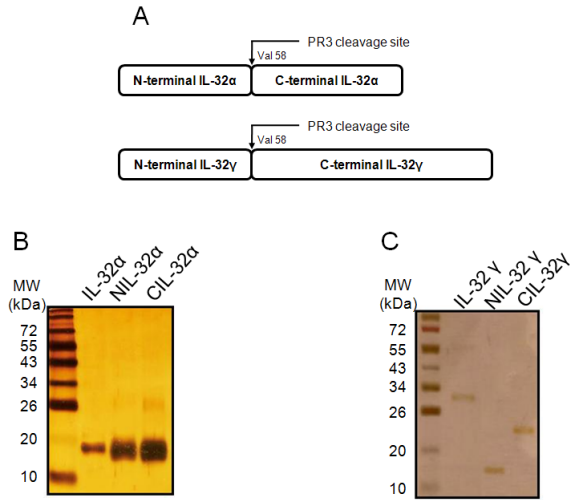

Fig. 1. Diagram of PR3-cleaved IL-32 separate domains and silver staining of the multistep-purified recombinant IL-32 separate domain. (A) The four PR3-cleaved IL-32 separate domains represent the PR3-cleaved N/C-termini of the four separate domains. The PR3-cleavage sites are indicated as valine 58 and 104 in isoform α and γ, respectively. (B) Four IL-32 separate domains including intrinsic isoform α and γ, as indicated above figures, were purified via multistep purification, subjected to 10% SDS-PAGE, and silver-stained in order to evaluate their purity.

RESULTS

Expression of the PR3-cleaved forms of IL-32 separate domains

A detailed diagram of the PR3-cleaved forms of the IL-32 separate domains is provided in Fig. 1A. The four PR3-cleaved forms of the IL-32 separate domains, N-terminal IL-32α (NIL-32α), C-terminal IL-32α (CIL-32α), N-terminal IL-32γ (NIL-32γ), and C-terminal IL-32γ (CIL-32γ), represent every possible PR3-cleaved form of the IL-32 isoforms. Four PR3-cleaved forms of IL-32 separate domain cDNAs were constructed, then inserted into pPROEX/HTa for expression in *E. coli*. A multistep purification (His^6-tag protein purification, size exclusion chromatography, and ion exchange chromatography) procedure was employed in order to obtain pure recombinant IL-32 separate domain protein from *E. coli*. The IL-32 proteins were subjected to 10%-SDS PAGE, and the purity of the four IL-32 separate domains and intrinsic IL-32 isoform α and γ were verified by silver staining (Fig. 1B and C). NIL-32α and CIL-32α (Fig. 1B) as well as NIL-32γ and CIL-32γ (Fig. 1C) exhibited a single band upon silver staining. However, NIL-32α and CIL-32α, in addition to CIL-32γ, migrated more slowly than intrinsic IL-32α and IL-32γ, respectively. Thus, the results of western blotting showed that the single band observed on the silver-stained gel was, indeed, recombinant IL-32 protein. The purified IL-32 separate domains were recognized specifically by rabbit anti-IL-32 polyclonal antibody (Fig. 2A), in addition to the anti-IL-32 monoclonal antibody clone 9 (Fig. 2B), both of which recognized all isoforms. As had been expected, the anti-IL-32 monoclonal antibody clone α7, which is specific for IL-32 isoform α, recognized only isoform α, including the PR3-cleaved forms of the N- and C-terminal IL-32α separate domains, although the Western band observed for the clone α7 IL-32α separate domain was far weaker than the intrinsic IL-32α isoform (Fig. 2C).

Bio-assay of four PR3-cleaved IL-32 separate domains

The bioactivities of multistep purified PR3-cleaved forms of IL-32 separate domains were assessed on freshly isolated human PBMC. Interestingly, NIL-32α and NIL-32γ-induced IL-1β

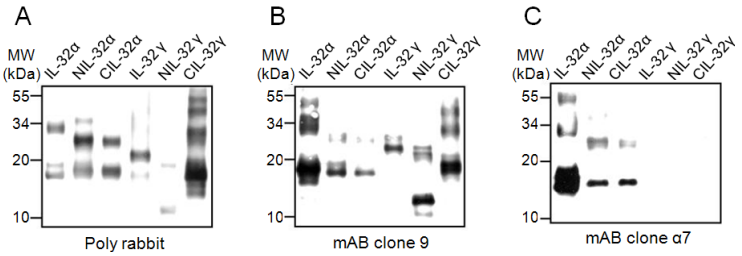

Fig. 2. Western blot of the four IL-32 separate domains and verification of their specificity with anti-IL-32α monoclonal antibody. Four IL-32 separate domain proteins (5 ng in each lane) and intrinsic isoforms α and γ were loaded and detected with (A) affinity-purified rabbit anti-IL-32α polyclonal antibody, (B) monoclonal antibody clone 9, which recognizes all isoforms, and (C) monoclonal antibody clone α7, which is specific for isoform α.

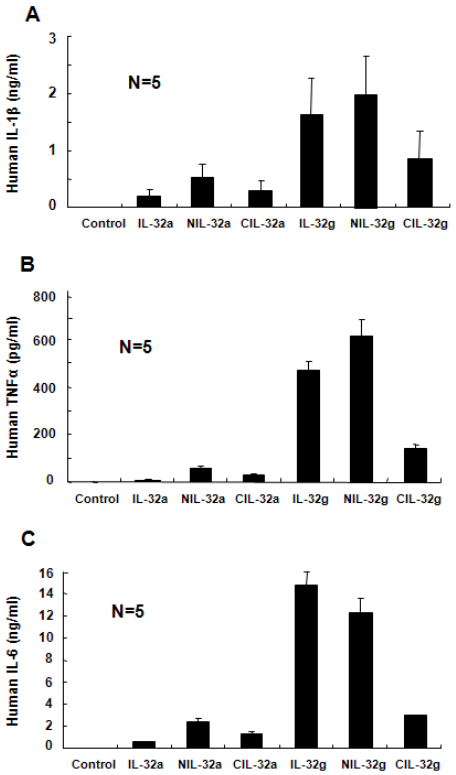

Fig. 3. The biological activity of the four IL-32 isoforms in human PBMC. Human PBMC were stimulated with each of the four IL-32 separate domains (50 ng/ml) or the intrinsic isoform α and γ in the presence of polymyxin B (100 U/ml) for 18 h, and the cell culture supernatants were then harvested for cytokine measurement. (A) IL-1β, (B) TNFα, and (C) IL-6 were measured by ECL.

and TNFα production occurred to a greater extent than was observed with intrinsic IL-32α and IL-32γ, respectively (Fig. 3A), whereas NIL-32 γ-induced less profound IL-6 production than was observed with intrinsic IL-32γ (Fig. 3C). CIL-32α, and CIL-32γ-induced cytokine production levels were far lower than the levels of NIL-32α and NIL-32γ Activity. In this assay, IL-32γ-induced cytokine production was greater than that induced by IL-32α. This is consistent with previous findings describing the cytokines induced by the four IL-32 isoforms (in press). In addition, we stimulated A549 human lung epithelial cells with PR3-cleaved forms of IL-32, as well as intrinsic forms, as lung inflammation is associated with neutrophil infiltration and neutrophil serine proteinases including PR3. Both forms interacted with A549 human lung epithelial cells, resulting in the production of human IL-8 (Fig. 4A). Intrinsic IL-32γ and the PR3-cleaved form of NIL-32γ induced IL-8 production 10 times that induced by IL-32α, similar to the PBMC data provided in Fig. 3A-C, whereas LPS, IL-18, and IFNγ did not react with A549 human lung epithelial cells (Fig. 3D).

Proposed model of lung inflammation associated with IL-32 and PR3 activities

The human respiratory system is vulnerable to a variety of airborne infectious pathogens, including viruses and bacteria. Infection and subsequent inflammation of the lung frequently occur, and represent one of the most serious diseases threatening human health. IL-32 and PR3, a neutrophil serine proteinase, may represent crucial players in the immune responses that occur during lung infection. As is shown in Fig. 4B, viral or bacterial infection stimulates the production of IL-32 from human lung epithelial cells. IL-32 is an inflammatory cytokine that induces IL-8, which induces chemotaxis in neutrophils,

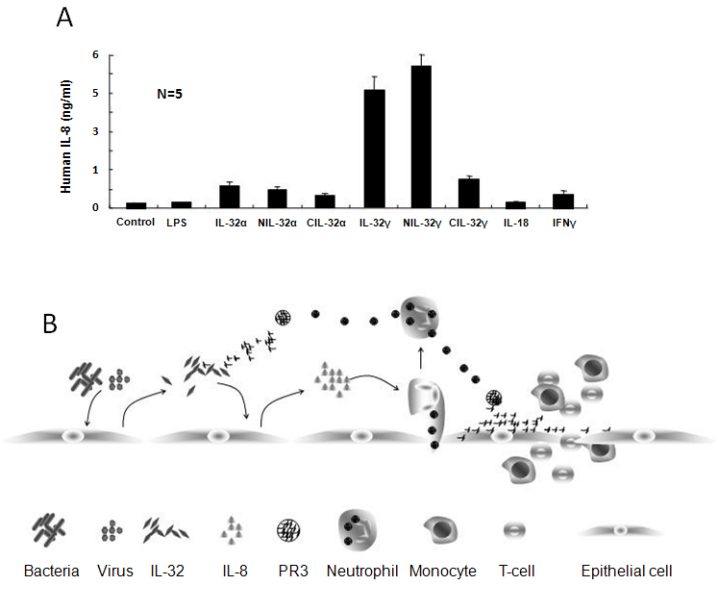

Fig. 4. Examination of IL-32 separate domain proteins with A549 human lung epithelial cells and the proposed model of lung inflammation associated with IL-32 and PR3 activities. (A) A549 human lung epithelial cells were stimulated with each of the four IL-32 separate domain proteins and the intrinsic isoforms α and γ as well as IL-18 and IFNγ, and IL-8 levels were measured in the cell culture supernatants after 18 hours of stimulation. (B) Bacterial and viral infections that occur in the human respiratory system induce IL-32, as well as other inflammatory cytokines. IL-32 stimulates lung epithelial cells to generate IL-8, recruiting neutrophils to the infected site. The infiltrating neutrophils release a large quantity of PR3, which amplifies IL-32 activity and initiates tissue damage and the further infiltration of secondary immune cells, including T-cells and monocytes.

thus recruiting them to the site of the infected area. The infiltrated neutrophils generate large quantities of PR3, which amplifies IL-32 activity and induces tissue damage, thereby resulting in the further infiltration of secondary immune response cells, including T-cells and monocytes.

DISCUSSION

Cytokine regulation is an important axis of the immune response for host defenses against the invasion of a variety of airborne pathogens into the respiratory system. The production of specific cytokines is crucial for developing a proper immune response to an antigen, and cytokine control is key to maintaining the homeostasis of immune responses. However, the uncontrolled upregulation of cytokines frequently results in tissue damage and chronic inflammatory disorders at the site of infection.

In the current study, we demonstrated the biological activity of PR3-cleaved recombinant IL-32 separate domains. The four IL-32 isoform genes were cloned originally from a human NK cell line and used as a template (2). PR3-cleaved recombinant IL-32 separate domains were generated and expressed to identify the most active form of the PR3-processed IL-32 separate domains. The multistep purification procedure yielded pure recombinant protein. The biological activity of each sample of purified IL-32 was then assessed. Interestingly, PR3-cleaved NIL-32γ induced slightly more IL-1β and TNFα than did intrinsic IL-32γ.

IL-32 mRNA transcripts exist as six different isoforms. IL-32 isoforms α, β, γ, and δ were the first to be described (2), and two more isoforms, ε and ζ, were recently described without detailed characterization (13). In human NK cells, the most abundant IL-32 isoform transcript was isoform α, which is the shortest isoform due to two additional splice sites in exons 3-4 and 7-8 (2). When four different recombinant IL-32 isoforms were expressed in *E. coli*, the IL-32 isoform α evidenced the highest levels of production, whereas the other three isoforms were poorly expressed as soluble proteins (data not shown). The expression of recombinant human IL-18 in *E. coli* is difficult, as it is prone to multimer formation, probably as the consequence of inter-disulfide bonding, thereby rendering it inactive (Fig. 2). A highly stable human recombinant IL-18 was generated via the replacement of the cysteine residue, which is not conserved among species, with serine, on the basis of the tertiary structure (14).

IL-32 specifically synergizes with the muropeptide agonist, which is common to all clinically relevant bacteria, for the nucleotide-binding oligomerization domains (NOD) 1 and 2. This synergism was observed in IL-6 production, although TNFα remained unaffected. Mutations in *NOD2* result in a significantly increased risk of developing Crohn's disease (15-17), and NOD2-deficient mice evidence increased susceptibility to lethality when infected with intracellular pathogens (18). The observed synergistic effect of IL-32 on NOD2 implies that this cytokine may perform a crucial role in Crohn's disease. It would be interesting to evaluate the effects of NOD2 on IL-32 activity in the respiratory system, as NOD2 has been shown to be crucial for mucosal immunity.

Assessing the biological activity of PR3-processed IL-32 separate domains *in vivo* is a difficult proposition, although it is crucial for the identification of the most active form of IL-32. Cytokine inhibition with a soluble receptor or antibody against the ligand or its receptor is an essential clinical approach for therapeutic purposes (19-21). Thus, it will be of great interest to determine the most active domain of IL-32 in order to elucidate a potential target for IL-32 inhibition, which would be beneficial for the treatment of chronic respiratory diseases.

The results of the current study demonstrate that the PR3-cleaved NIL-32γ separate domain exerts the most significant levels of activity, inducing a variety of proinflammatory cytokines including IL-1β, IL-6, and TNFα in human PBMC and IL-8 in A549 human lung epithelial cells. It is possible that distinct profiles of IL-32 isoform regulation, resulting in additional effects of PR3, occur in different individuals. For example, a certain individual may express the most active form of IL-32, consequently developing chronic inflammatory disorders. Thus, it will be important to characterize which of the PR3-cleaved IL-32 products is the most active form of IL-32 in inflammation and immunity.

MATERIALS AND METHODS

Reagents

In an effort to isolate rabbit anti-IL-32 IgG antibodies, the DEAE blue affi-gel column was obtained from BioRad (Hercules, CA). Affi-15 agarose beads were purchased from BioRad in order to generate the IL-32α ligand-affinity column. TALON affinity beads, Tobacco Etch Virus (AcTEV) protease, and pPROEX/HT *E. coli* expression vector were obtained from Invitrogen (Carlsbad, CA). The TA cloning vector pGEMT Easy was obtained from Promega (Madison, WI).

Construction of PR3-cleaved IL-32 separate domains for *E. coli* expression

We cloned the IL-32 splice variants, and then the open reading frames of the IL 32 isoform α and γ cDNAs were transferred into the *E. coli* pPROEX/HTa expression vector (Invitrogen) using the EcoRI and XabI restriction enzyme sites (11). The *E. coli* expression vectors for PR3-cleaved IL-32 separate domains were constructed in accordance with our previous report (5). In brief, NIL-32α and NIL-32γ were generated via the addition of a stop codon (TGA) after the amino acid sequence "GHLET," and CIL-32α and CIL-32γ were generated by fusing the amino acid sequence "VAAYY" to the His^6-tag of the pPROEX/HTa vector.

Expression and purification of recombinant IL-32 separate domain proteins

Four PR3-cleaved IL-32 separate domain proteins, N/C-terminal

IL-32α (N/CIL-32α) and N/C-terminal IL-32γ (N/CIL-32γ), were expressed in *E. coli* and purified with a TALON affinity column (Invitrogen) via the His^6-tag at the N-terminus of the proteins. The TALON affinity-purified proteins were subjected to size exclusion chromatography (Superdex 75, ÄKTA*FPLC*), then digested with TEV protease (Tobacco Etch Virus, Invitrogen) for 16 h at 4°C in order to remove the His^6-tag from the fusion protein. The AcTEV cleaved proteins were dialyzed in Tris-base buffer (50 mM, pH 8), then subjected to ion exchange chromatography (HiTrapQFF, ÄKTA*FPLC*). Each purified IL-32 separate domain protein was assessed for biological activity.

Production and purification of poly and monoclonal antibodies for Western blotting

A polyclonal antibody generated in rabbits against IL-32α were utilized for Western blotting. Rabbit anit-IL-32α polyclonal antibody was generated at Rockland (Gilbertsville, PA) in accordance with the manufacturer's recommendations. The Anti-IL-32α IgG fraction was isolated using the DEAE blue affi-gel column, and then the IgG fraction was further affinity-purified with an IL 32α-immobilized agarose bead column containing Affi gel 15. The anti-IL-32 monoclonal antibody clones 9 and α7 were generated as described (12). The recombinant proteins were subjected to 10% SDS-PAGE, then transferred to a nitrocellulose membrane. The membrane was probed with affinity purified rabbit anti-IL-32α polyclonal antibody or monoclonal antibodies (0.5 μ/ml in PBS containing 5% skim milk and 0.05% Tween 20) for 18 h, followed by the secondary antibody (rabbit anti-rabbit IgG-HRP). The Western blotting membrane was analyzed via Bio-imaging using the LAS-4000 (Fujifilm Lifescience USA, Stamford, CT).

Cell culture and cytokine assays

Human peripheral blood mononuclear cells (PBMC) were isolated via the density centrifugation of blood diluted 1:1 in pyrogen-free saline over Histopaque (Sigma, St. Louis, Missouri). PBMCs were washed twice with culture medium (RPMI 1640) containing penicillin (100 U/ml), streptomycin (100 U/ml), and glutamine (29.2 μg/ml) and resuspended in the same culture medium.

PBMC (5×10^5) were seeded in 96-well plates in a volume of 100 μl, then incubated with either 100 μl of culture medium (control) or 100 μl of medium containing one of the following stimuli: LPS (20 ng/ml): 100 ng/ml of each NIL-32α, CIL-32α, NIL-32γ, and CIL-32γ. After 18 h of treatment with various stimuli, the cell culture supernatants were evaluated for cytokine levels. The liquid phase ECL method was utilized to measure human IL-1β, IL-6, and TNFα as well as mouse MIP 2 and TNFα in the cell culture media and serum samples (BioVeris, Gaithersburg, MD). The electrochemiluminescence levels were determined using an Origen Analyzer (BioVeris).

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