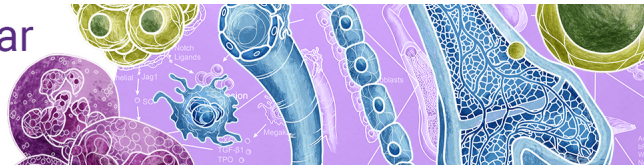


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Cutting Edge: CIAS1/Cryopyrin/PYPAF1/NALP3/ CATERPILLER 1.1 Is an Inducible Inflammatory Mediator with NF- κ B Suppressive Properties

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CUTTING EDGE

Cutting Edge: CIAS1/Cryopyrin/PYPAF1/NALP3/CATERPILLER 1.1 Is an Inducible Inflammatory Mediator with NF- κ B Suppressive Properties

William O'Connor, Jr., Jonathan A. Harton, Xinsheng Zhu, Michael W. Linhoff, and Jenny P.-Y. Ting¹

Mutations in the cold-induced autoinflammatory syndrome 1 (CIAS1) gene have been recently linked to three chronic autoinflammatory disorders. These observations point to an important role for CIAS1 in regulating inflammatory processes. We report that TNF- α and ligands recognized by multiple Toll-like receptors rapidly induce CIAS1 gene expression in primary human monocytes. Transfection of full-length CIAS1 or either of two shorter, naturally occurring isoforms dramatically inhibited TNF- α -induced activation of NF- κ B reporter activity. Furthermore, CIAS1 suppressed TNF- α -induced nuclear translocation of endogenous p65. Transcriptional activity of exogenous NF- κ B p65 was also blocked by CIAS1. The nucleotide-binding and leucine-rich repeat regions, but not the pyrin domain of CIAS1, are responsible for this inhibition. These data suggest CIAS1/cryopyrin may act as a key regulator of inflammation, induced to dampen NF- κ B-dependent proinflammatory signals. The Journal of Immunology, 2003, 171: 0000–0000.

The cold-induced autoinflammatory syndrome 1 (CIAS1) gene belongs to the CATERPILLER (CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats), family described previously (1) which consists of >20 genes implicated in regulating apoptosis or inflammation. Before that report, subgroups of this family have also been named NBD/LRR (nucleotide-binding domain and leucine-rich repeat), NACHT (NAIP, CIITA, HET-E, and TP-1), or PYPAF (pyrin-containing Apaf-1-like proteins), categorized by different criteria.

The locus responsible for the chronic, autosomal-dominant autoinflammatory periodic fever syndromes familial cold urticaria and Muckle-Wells syndrome was found on chromosome 1q44 with pathology-associated mutations present in the CIAS1 gene (2). Common symptoms of these genetic disorders

include periodic fever, rash, arthralgia, and conjunctivitis. Mutations in CIAS1 were also found to be associated with the chronic infantile neurologic, cutaneous, articular syndrome (3). CIAS1 encodes the protein first named cryopyrin (2), also termed PYPAF1/NALP3/CATERPILLER 1.1 subsequently reported by other groups including our own (1, 4, 5).

CIAS1 contains an amino-terminal pyrin domain, a centrally located predicted NBD, and numerous LRR motifs at its carboxyl terminus. The function of each domain is largely unknown. The pyrin domain of CIAS1 is highly homologous to its namesake, the Pyrin protein encoded by the MEFV gene (1). Recent published reports provide evidence that CIAS1 may be involved in the regulation of IL-1 generation and NF- κ B activation (4, 6), placing CIAS1 in the inflammatory cascade.

The inflammatory signaling program in myeloid cells leads to activation of the cytokines IL-1, IL-6, IL-8, and TNF- α , as well as reactive oxygen species and other molecules through a number of critical steps culminating in transcriptional activity (reviewed in Ref. 7). Initiation of the signaling cascade frequently begins with cell surface-expressed TLRs sensing a variety of pathogenic products, stimulation of the IL-1R, or cross-linking of the TNF- α receptor. These diverse signaling pathways initially utilize an assortment of signaling intermediates (7–9) but converge downstream to induce activity of the transcription factors NF- κ B, AP-1, and others.

TNF- α stimulation leads to phosphorylation, ubiquitination, and degradation of I κ B α , liberating the p50 and p65 subunits of NF- κ B. The p65 subunit is then phosphorylated and enters the nucleus to initiate transcription of various inflammatory genes. Since TNF- α is widely regarded as one of the most potent inflammatory stimulants, we focus on the effects of CIAS1 on TNF- α signaling in this report. We began by cloning several isoforms of the CIAS1 gene. Concurrent with our isolation of the gene, others formally reported two of these sequences (2, 4). In this study, we first examined the inducible

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² Abbreviations used in this paper: CIAS1, cold-induced autoinflammatory syndrome 1; CATERPILLER, CARD, transcription enhancer, R(purine)-binding, pyrin, lots of leucine repeats; LTA, lipoteichoic acid; NBD, nucleotide-binding domain; LRR, leucine-rich repeat; ASC, apoptosis-associated speck-like protein; PYPAF, pyrin-containing Apaf-1-like protein.

nature of *CIAS1* in human monocytes and then assessed downstream consequences of its presence.

Materials and Methods

Monocyte preparation and real-time PCR analysis

Primary human monocytes were isolated from normal donor buffy coat preparations (American Red Cross). PBMCs were obtained by standard Ficoll centrifugation procedure. The nonadherent fraction was removed and fresh medium was then added alone or with stimulant as indicated. LPS from *Escherichia coli* (LPS 026:B6; Sigma-Aldrich, St. Louis, MO) or *S. enteritidis* (Sigma-Aldrich) was added to 200 ng/ml; lipoteichoic acid (LTA from *Staphylococcus aureus*; Sigma-Aldrich) added to 1 μ g/ml; poly(I:C) (Amersham, Arlington Heights, IL) to 10 μ g/ml; and CpG oligonucleotide (ODN 1668) or control GpC oligonucleotide (ODN 1720, both from TIB MolBio) to 1 μ M. TNF- α was added to 50 ng/ml. Cells were stimulated for 1 h at 37°C except where indicated. RNA was isolated as per the manufacturer's instructions (SV Total RNA Isolation; Promega, Madison, WI) and first-strand synthesis was performed (Moloney murine leukemia virus-reverse transcriptase).

Real-time PCR analyses were performed on the Applied Biosystems Prism 7700 instrument (Applied Biosystems) (10). *CIAS1* gene expression was determined using the following intron-spanning primers for amplification: forward primer, 5'-GGCATATCACAGTGGGATTC-3'; reverse primer, 5'-GATCTTCGCTGCGATCAAC-3'. Amplification of 18S RNA was performed as described previously (10). *CIAS1* expression was quantitated by comparing values obtained to a standard curve generated with plasmid DNA. All *CIAS1* values obtained were normalized to 18S RNA (*CIAS1* molecules/attomole of 18S) and reported as differences in fold induction of *CIAS1* over levels of *CIAS1* in untreated, resting monocyte cultures.

Ab production and Western blot procedure

Rabbit antiserum was generated to the synthetic polypeptide MASTRCK LARYLEDLEDVLDLKK corresponding to the amino terminus of human *CIAS1*/cryopyrin. The 22-mer was conjugated to keyhole limpet hemocyanin and immunization was performed as per standard protocol (ProteinTech). Primary human monocytes were supplemented with RPMI 1640 medium or medium with *E. coli* LPS (1 μ g/ml) for the times indicated before lysis with radioimmunoprecipitation assay buffer (150 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Nonidet P-40, 50 mM Tris (pH 8.0)). Equivalent quantities of protein were subjected to SDS-PAGE through 10% polyacrylamide and transferred to nitrocellulose membranes as per standard protocol. Membranes were incubated with rabbit polyclonal anti-*CIAS1* or mouse anti-actin for 1 h and visualized with goat anti-rabbit or goat anti-mouse HRP-conjugated secondary Abs (Santa Cruz Biotechnology, Santa Cruz, CA). Detection was performed with SuperSignal Chemiluminescence Reagents (Pierce, Rockford, IL).

Cell transfection and luciferase assays

HeLa cells (American Type Culture Collection, Manassas, VA) were transfected with the indicated quantities of the following FLAG-tagged *CIAS1* constructs: full-length wild-type (Fg*CIAS1*), *CIAS1* deletion exon 4 (Fg Del4, GenBank accession AY422168), *CIAS1* deletion exons 4 and 6 (Fg Del4 Del6), *CIAS1* truncation mutants (*CIAS1* pyrin, *CIAS1* pyrin/NBD, *CIAS1* NBD/LRR, or *CIAS1* LRR), or pcDNA3 along with 100 ng of 3 \times -NF- κ B-luciferase using Fugene6 (Roche, Basel, Switzerland). Twenty-four hours posttransfection, cells were stimulated with TNF- α (10 ng/ml) or transfected with either empty vector or pCMV4T-p65 (500 ng/well) and incubated at 37°C for another 24 h. Cells were then lysed and luciferase was quantitated as per standard protocol. The p53-luciferase control reporter construct was obtained from Dr. Y. Xiong (University of North Carolina, Chapel Hill) and used at 500 ng/well.

Immunofluorescent staining and quantitation

HeLa cells were transfected with 1.5 μ g/well FLAG-*CIAS1* or pcDNA3 (Fugene 6). Twenty-four hours after transfection, cells were stimulated with TNF- α (10 ng/ml) or medium alone for 30 min at 37°C. Indirect immunostaining was performed by standard protocol. Endogenous p65 was visualized using a rabbit anti-p65 Ab (Santa Cruz Biotechnology) and goat anti-rabbit-biotin/avidin-Texas Red secondary Abs (Vector Laboratories, Burlingame, CA). FLAG-tagged *CIAS1* was visualized using the FLAG Ab M5 (Sigma-Aldrich) and goat anti-mouse FITC (BD Pharmingen, San Diego, CA). Nuclei were counterstained using 4',6'-diamidino-2-phenylindole (Vector Laboratories). The subcellular localization of endogenous p65 was assessed in 100 *CIAS1*^{low/neg} cells and compared with p65 in 100 *CIAS1*^{high} cells in three double-blind studies.

Results and Discussion

CIAS1 is induced by stimulants of Toll-like receptor (TLR) signaling

Expression of *CIAS1* in peripheral blood cells was determined to be primarily restricted to monocytes (our unpublished observation and Ref. 4). To determine whether *CIAS1* is inducible in primary human monocytes, we used real-time PCR analysis to quantitate levels of *CIAS1* mRNA in both resting and activated cells. LTA, poly(I:C), LPS, and CpG oligonucleotides are well characterized stimulants of TLR-2, -3, -4, and -9, (11–14). Administration of LTA, LPS, or poly(I:C) to primary human monocytes elicited a robust induction of *CIAS1* expression (Fig. 1A). LTA and poly(I:C) preparations were found to be free of contaminating endotoxin (<0.06 endotoxin units/ml) as determined by the *Limulus* amoebocyte assay. No changes in *CIAS1* gene expression were seen with CpG oligonucleotides (data not shown), likely due to the absence of TLR9 on human monocytic cells.

E. coli LPS acted rapidly to induce an approximate 15-fold increase in *CIAS1* expression within 30 min of stimulation, with expression reaching >20-fold by 1 h (Fig. 1B). Similarly, TNF- α induced a >10-fold increase in *CIAS1* gene expression during the same period (Fig. 1C). The levels of *CIAS1* gene expression with LPS and TNF- α were reproducible within experiments and between multiple donor blood preparations. The expression of *CIAS1* is likely under tight regulation as *CIAS1* mRNA is low in resting monocytes, induced strongly within 1 h of TNF- α or LPS stimulation, and returns to baseline levels within 3–6 h poststimulation (Fig. 1C and our unpublished observations).

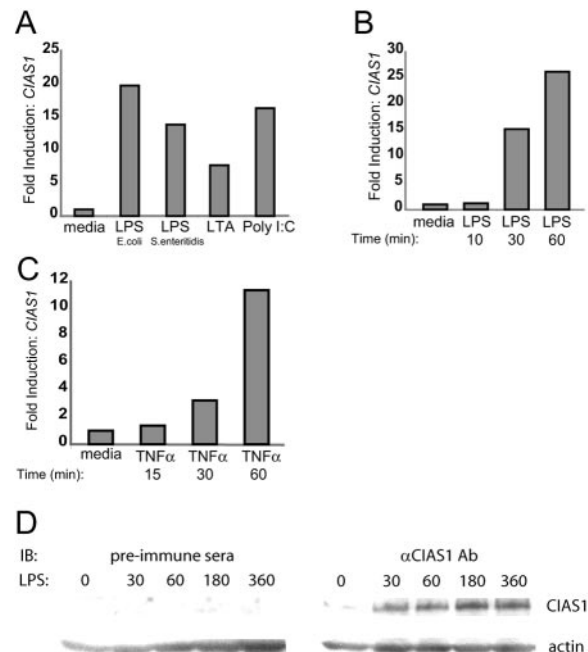


FIGURE 1. Induction of *CIAS1* in primary human monocytes. *A*, Adherence-purified human monocytes were stimulated as indicated for 1 h before lysis, preparation, and analysis. LPS (*B*) and TNF- α (*C*) rapidly induce *CIAS1*. Results are representative of three or more assays performed in triplicate on cDNAs generated from multiple donor blood preparations. *D*, *CIAS1* protein levels were examined in LPS-stimulated primary human monocytes for up to 3 h (shown in minutes) using polyclonal rabbit antiserum raised to *CIAS1*.

To examine changes in CIAS1 protein levels, we generated a polyclonal Ab to the amino terminus of human CIAS1. Primary human monocytes were treated with LPS from *E. coli* over a 3-h period and whole-cell lysates were subjected to SDS-PAGE. Endogenous full-length CIAS1, detected near the predicted 120 kDa, is seen at low levels in resting monocytes and is induced within the first few hours of LPS stimulation (single donor, Fig. 1*D*). The induction of CIAS1 protein varied between individual donors, ranging from individuals displaying higher basal levels of CIAS1 to those with barely detectable or undetectable protein levels. Thus, CIAS1/crocyrin is in low abundance at both the mRNA and protein levels and induced with inflammatory stimuli.

Multiple isoforms of CIAS1 inhibit NF- κ B reporter activity

The rapid induction of *CIAS1* by immunostimulatory molecules led us to hypothesize that CIAS1 may play a role in mediating the inflammatory response. Since NF- κ B activity has been intimately linked to inflammation (reviewed in Ref. 15), we analyzed NF- κ B activity in the presence of transfected *CIAS1*. Transfection of full-length *CIAS1* (Fig. 2*A*) did not lead to activation of NF- κ B-luciferase. Additionally, we have isolated two shorter, naturally occurring splice variants of *CIAS1*, one of which is novel (del4). They also did not activate the NF- κ B luciferase reporter. Surprisingly, careful examination of the data set revealed relative decreases in basal NF- κ B-luciferase activity in the *CIAS1*-positive lanes (our unpublished observation). TNF- α activates NF- κ B and, in our experiments, TNF- α elicited NF- κ B reporter activity as expected (Fig. 2*B*, first two lanes). Since the observed decreases in basal activity suggested a possible inhibitory role for CIAS1, we tested the ability

of CIAS1 to inhibit TNF- α -induced NF- κ B-luciferase. As shown in Fig. 2*B*, expression of all three *CIAS1* isoforms led to a strong, dose-dependent inhibition of TNF- α -induced NF- κ B-luciferase. The inhibition seen was not due to interference with or dysregulation of endogenous CIAS1, as HeLa cells do not express endogenous *CIAS1* with or without LPS or TNF- α addition (undetectable mRNA, even at 40 cycles of amplification, data not shown).

Many signaling pathways leading to the activation of NF- κ B share a common mechanism of action that liberates the p50 and p65 subunits of NF- κ B from the I κ B complex, allowing them to be phosphorylated and imported into the nucleus. To identify the position CIAS1 occupies in the NF- κ B pathway, we analyzed the effects of CIAS1 on p65 induction of the NF- κ B-luciferase construct. CIAS1 dramatically inhibited the ability of p65 to activate the NF- κ B-luciferase reporter in a dose-dependent fashion (Fig. 2*C*). This indicates that CIAS1 functions at the distal end of NF- κ B signaling by affecting p65 function. p53 induction of a p53-responsive luciferase construct was largely unaffected, reflecting the specificity of CIAS1. Additionally, a hemagglutinin-tagged *CIAS1* showed identical results (data not shown).

CIAS1 functions in the cytoplasm

Indirect immunofluorescence studies were performed to visualize the subcellular localization of overexpressed CIAS1. Full-length CIAS1 localizes to the cytoplasm in the absence of any stimulus (4), but the effects of cellular stimulation on the localization of CIAS1 are not known. To assess this, we transiently transfected HeLa cells with the concentration of *CIAS1* shown to inhibit NF- κ B-luciferase and visualized FLAG-tagged

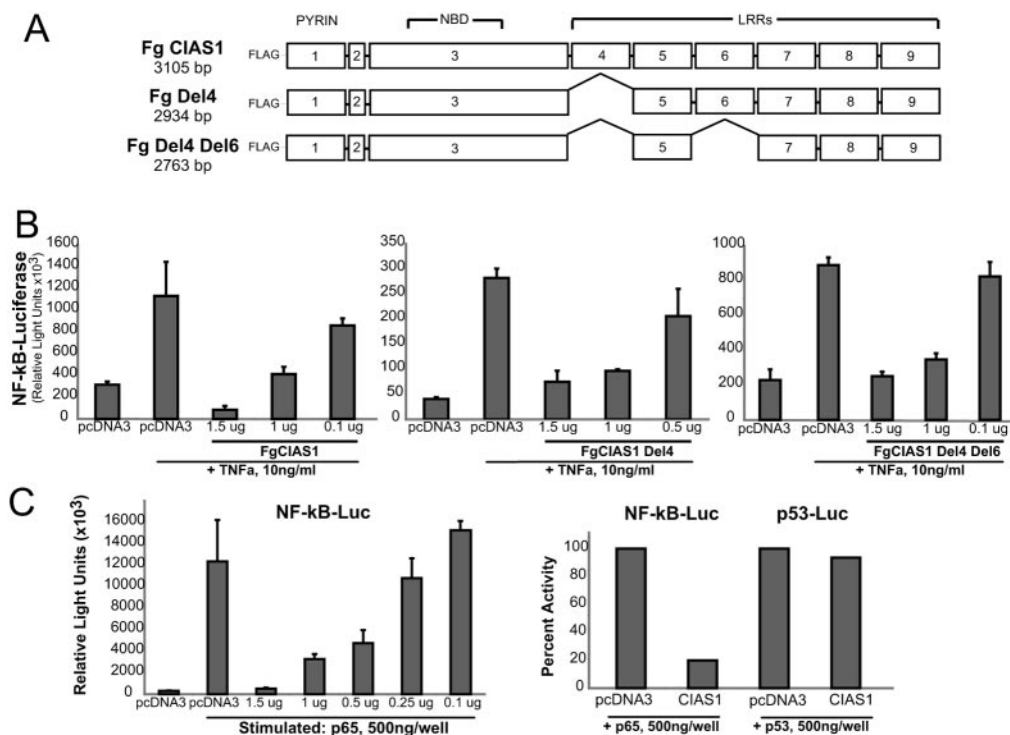


FIGURE 2. CIAS1 inhibition of TNF- α and p65-induced NF- κ B-luciferase. FLAG-tagged full-length *CIAS1* (*FgCIAS1*) and the shorter, naturally occurring isoforms missing exon 4 or exons 4 and 6 are depicted in *A*. All of the constructs in *A* inhibited NF- κ B-luciferase activation by TNF- α in a dose-dependent fashion (*B*). *FgCIAS1* inhibited the ability of transfected p65 to stimulate NF- κ B-luciferase in a dose-dependent fashion (*C*, left panel). *FgCIAS1* did not affect p53 function (*C*, right panel). Data are representative of three or more assays performed in triplicate \pm SEM.

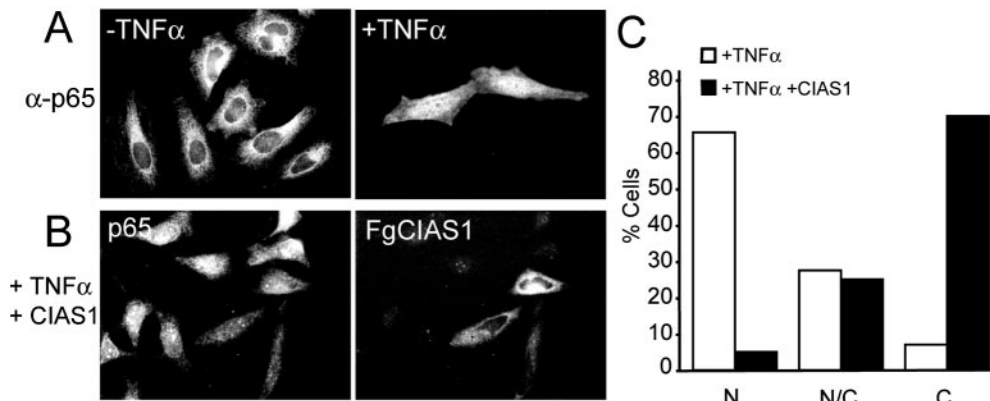


FIGURE 3. *FgCIAS1* inhibits TNF- α -induced nuclear translocation of p65. Localization of endogenous NF- κ B p65 in untreated or TNF- α -stimulated HeLa cells is shown in *A*. *FgCIAS1* markedly reduced nuclear accumulation of endogenous p65 in response to TNF- α (*B*). *CIAS1*-positive cells are identified (*B*, right panel) and show reduced nuclear p65 (p65 visualized in *B*, left panel). Nuclei were counterstained with 4',6'-diamidino-2-phenylindole (data not shown). Localization of p65 in *CIAS1*-positive cells was scored qualitatively (*C*) as described in *Materials and Methods*. p65 localization was scored as primarily nuclear (N), evenly nuclear/cytoplasmic (N/C), or primarily cytoplasmic (C) p65. Quantitative data shown are the composite percentages from three individual experiments.

CIAS1. Twenty minutes of TNF- α stimulation potently induced endogenous p65 to enter the nucleus (Fig. 3*A*) but did not lead to nuclear translocation of *FgCIAS1* (Fig. 3*B*, right). These data suggest that *CIAS1* functions in the cytoplasm to inhibit NF- κ B.

CIAS1 inhibits nuclear translocation of p65

Inhibition of NF- κ B can occur at any of several stages in the activation cascade. The observation that *CIAS1* inhibits exogenously transfected “free” p65 suggested to us that one function of *CIAS1* might be to inhibit nuclear translocation of the p65 subunit. To test this, we transiently transfected HeLa cells with *CIAS1* and analyzed TNF- α -induced nuclear translocation of endogenous p65. TNF- α stimulation of NF- κ B leads to rapid movement of p65 into the nuclear compartment (Ref. 16 and Fig. 3*A*). In the presence of *CIAS1*, a significant reduction in the amount of nuclear p65 was seen in response to TNF- α (Fig. 3*B*). A double-blind numerical analysis of this effect was performed (Fig. 3*C*).

Inhibition of TNF- α signaling is mediated by the nucleotide-binding and leucine-rich repeat regions of CIAS1

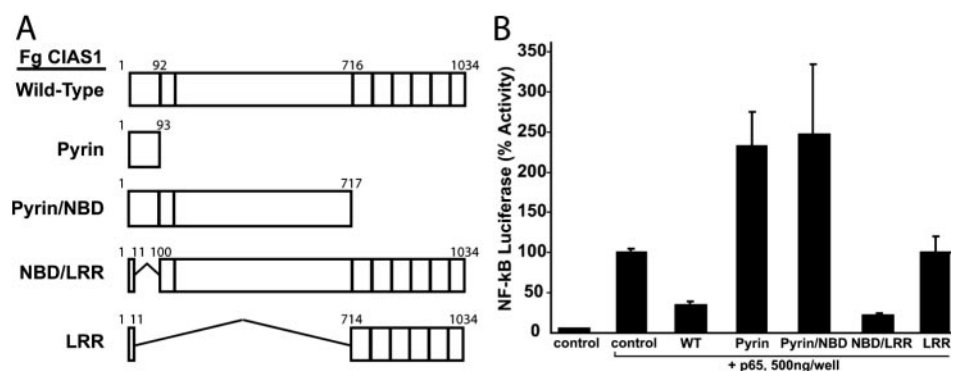
To better understand the inhibitory nature of *CIAS1* in TNF- α signaling, a series of FLAG-tagged deletion constructs of *CIAS1* were generated (Fig. 4*A*) and tested. The NBD and LRR regions together inhibited TNF- α -induced NF- κ B activity as well as the full-length construct (Fig. 4*B*). Thus, deletion of the

amino-terminal pyrin domain had no deleterious effect on inhibition. Curiously, transfection of pyrin-containing truncation mutants cooperatively activated the NF- κ B reporter in the presence of p65 (Fig. 4*B*).

The onset of inflammation is a central response to pathogens, autoimmune Ags, and injury. Yet, the resolution and down-regulatory phase of this response to prevent irrevocable damage is of equal importance. This study shows that *CIAS1* is induced by a variety of proinflammatory stimuli including both cytokine and TLR agonists, seemingly to act as a negative regulator of NF- κ B signaling. This inhibition is concentration dependent and occurs, at least in part, by disallowing nuclear translocation of the p65 subunit of NF- κ B. That *CIAS1* may lead to a decrease in total cellular p65 remains possible; further studies are needed to understand the precise role played by *CIAS1*. Rather than leading to a reduction of cellular p65, we favor the hypothesis that *CIAS1* may disrupt activation of NF- κ B by interfering with a critical binding partner or upstream kinase.

Previously, *CIAS1* has been suggested to play a role in the generation of IL-1 and activation of NF- κ B, but only when expressed in concert with the adaptor molecule apoptosis-associated speck-like protein (ASC) (4). During the preparation of this manuscript, a report confirmed that the combination of *ASC* and *CIAS1* causes the activation of NF- κ B, but additionally shows that *ASC* alone had the opposite effect by inhibiting NF- κ B activation (17). Similar to these findings with *ASC*, our

FIGURE 4. Analysis of *CIAS1* domains regulating p65-induced NF- κ B-luciferase. Deletion mutant constructs of *CIAS1* are depicted in *A*. Relative activation of NF- κ B luciferase in HeLa cells transfected with 1.5 μ g/well pcDNA3 (control) or the indicated construct, followed by transfection with p65 24 h later as described in *Materials and Methods* (*B*). Values are means of three experiments performed in triplicate \pm SEM. WT, Wild type.



data reveal that CIAS1 alone reduces TNF- α and NF- κ B responses. It would be interesting to ascertain whether ASC or CIAS1 alone utilize similar or differing mechanisms of action to inhibit NF- κ B. Taken together, these studies suggest that the balance of ASC and CIAS1 critically determine the extent of inflammatory responses and that alone, either may serve as an important suppressor molecule. It is interesting to note that NF- κ B nuclear translocation is routinely detectable within 10–30 min after cell activation while increases in *CIAS1* mRNA are observed 30–60 min after stimulation. One possibility is that CIAS1 is induced to limit the extent of proinflammatory cytokine production, preventing hyperinflammation seen in autoinflammatory syndrome patients. In this scenario, mutations in *CIAS1* could lead to dysfunctional inhibition and prolonged, exaggerated inflammatory responses.

Other proteins with similar CARD and/or pyrin domains have been shown to activate NF- κ B in vitro. One example is Nod1, proposed to induce NF- κ B activity by bringing the CARD-containing kinase RICK in close proximity with the γ regulatory subunit of I κ B kinase (18). Another report describes a complex of CARD- and pyrin-containing proteins assembling to elicit processing of pro-IL-1 β , a signaling platform termed the “inflammasome” (19). In contrast, the CARDINAL/TUCAN and PAN2 proteins possess NF- κ B suppressor activity (20, 21). The emerging view is of a complex balance between proinflammatory and anti-inflammatory molecules that in the proper context serve to initiate, amplify, or suppress inflammatory processes.

The inhibitory activity of CIAS1 is dependent on the predicted NBD and LRR regions. LRR motifs appear in plant disease-resistance genes and may directly bind pathogenic substrate (for review, see Ref. 22). It is tempting to speculate that LRRs operate similarly in humans; however, to date, no truly convincing data regarding this hypothesis have been put forward. How LRRs together with a predicted NBD might mediate inhibition of NF- κ B signaling remains unclear. A curious finding is the stimulatory activity of the CIAS1 pyrin domain alone. We speculate that the pyrin domain expressed alone may artificially act as an oligomerization domain, bringing NF- κ B-activating molecules together as has been proposed for Nod1. In addition, positive cooperation of CIAS1 with ASC also involves the pyrin domain (4).

In conclusion, CIAS1 regulation of NF- κ B activity may have dramatic effects on myeloid cell growth, death, and/or inflammatory cytokine production. This regulation is context dependent, similar to recent findings for ASC. When expressed without ASC, CIAS1 appears to be a negative regulator of inflammation that may be important for the resolution of inflammatory responses.

Note added in proof. Two reports were published during the preparation of this manuscript showing Nod1 to bacterial components (23, 24). These data lend support for the hypothesis that NBD-LRR proteins may directly bind pathogenic substrate.

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