Identification of a ZU5 and Death Domain-containing Inhibitor of NF-κB*

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The transcription factor NF-κB plays important roles in inflammation and cell survival. NF-κB is composed of homodimeric and heterodimeric complexes of Rel/NF-κB family members, including p65 (RelA), c-Rel (Rel), RelB, NF-κB1/p50, and NF-κB2/p52. Here we report the identification and characterization of a novel ZU5 and death domain-containing protein designated ZUD. In reporter gene assays, overexpression of ZUD inhibited NF-κB-dependent transcription induced by both tumor necrosis factor (TNF) and interleukin-1 and their downstream signaling proteins. Gel shift assays indicated that the overexpression of ZUD inhibited binding of NF-κB to its target sequence. ZUD is a cytoplasmic protein, and coimmunoprecipitation assays indicated that ZUD interacted with the NF-κB subunit p105 and transactivator p65. Consistent with its role in inhibition of NF-κB-dependent transcription, ZUD sensitized cells to apoptosis induced by TNF and the TNF-related apoptosis-inducing ligand (TRAIL). Our findings suggest that ZUD is an inhibitor of NF-κB activation and that this protein may provide an alternative regulatory mechanism for NF-κB-mediated transcription.

The NF-κB/Rel family of transcription factors play critical roles in the regulation of immune responses, inflammation, apoptosis, and cell survival through induction of a large set of downstream genes, including cytokines, chemokines, adhesion molecules, and effectors (1–7). Five members of this family have been identified in vertebrates, including p65 (RelA), c-Rel (Rel), RelB, NF-κB1/p50, and NF-κB2/p52. All of these proteins share a conserved 300-amino acid region known as the Rel homology domain, which is responsible for DNA binding, dimerization, and nuclear translocation of NF-κB. These proteins bind DNA as homo- and hetero-dimers. Two of the active subunits of NF-κB, p50 and p52, are derived from the N-terminal domains of their precursor molecules, p105 and p100, respectively, via post-translational processing of the C-terminal ankyrin repeat-containing domains by the 26 S proteasome (1, 2). The major cellular form of NF-κB is a heterodimer consisting of the DNA binding subunit p50 and the transactivator p65. Normally, NF-κB is retained in the cytoplasm of unstimulated cells through association with its inhibitor IκB.

Upon stimulation by various NF-κB activating signals, IκB is phosphorylated and degraded through an ubiquitin-dependent process. This process frees NF-κB, which is then translocated into the nucleus to activate transcription of its target genes (1–7). The transcriptional activity of NF-κB is also modulated by regulation of p65 phosphorylation (4, 6).

TNF is one of the major proinflammatory cytokines that activate NF-κB (8). Upon TNF stimulation, TNF receptor 1 (TNF-R1), which contains a cytoplasmic death domain (DD), trimerizes and recruits the downstream DD-containing protein TRADD (TNF-receptor-associated death domain) to the receptor signaling complex (9, 10). TRADD then functions as an adaptor to recruit several downstream proteins, including FADD (Fas-associated death domain), TRAF2 (TNF-receptor-associated factor 2), and RIP (receptor-interacting protein) to the TNF-R1 signaling complex (9–12). Although FADD is critically involved in TNF-R1-mediated apoptosis, TRAF2 and RIP recruit IKK (IκB kinase) to the TNF-R1 complex (13, 14). IKK contains three subunits, the catalytic subunits IKKe and IKKβ and the regulatory subunit IKKγ (6). Once IKK is recruited to the TNF-R1 complex, it is activated and subsequently leads to IκB phosphorylation and NF-κB activation (6, 7).

Several members of the TNF receptor family contain a conserved DD that is essential for the NF-κB activation and apoptosis induced by these receptors (15). The DD is also found in several cytokolic adapted proteins, including TRADD, FADD, and RIP, and the NF-κB subunits p105 and p100 (15–20). The death domain exerts its effects via self-association and/or interaction with death domain of other proteins (15).

Here we report the cloning and characterization of a novel DD-containing protein designated ZUD (ZU5 and death domain-containing protein). In addition to the C-terminal DD, ZUD also contains an N-terminal ZU5 domain that is found in the cytoplasmic domains of UNC5H receptor family members (21–26). Our data demonstrate that ZUD is a cytoplasmic protein associated with p105 and p65 and inhibits NF-κB-dependent transcription.

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1 The abbreviations used are: TNF, tumor necrosis factor; DD, death domain; EST, expressed sequence tag; FADD, Fas-associated DD protein; IFN, interferon; IKK, inhibitory κB kinase; IL-1, interleukin 1; IRF-1, interferon response factor 1; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; RIP, receptor-interacting protein; SINK, p65-interacting inhibitor of NF-κB; TNF-R1, TNF receptor 1; TRAF, TNF receptor-associated factor; TRADD, tumor necrosis factor receptor-associated DD protein; TRAIL, TNF-related apoptosis-inducing ligand; UNC5H, Caenorhabditis elegans UNC-5 homolog; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; ZUD, ZU5 and death domain-containing protein.
Northern Blot Hybridization—Human multiple tissue mRNA blots were purchased from Clontech (Palo Alto, CA). The cDNA probe was an 1.6-kb fragment corresponding to the coding sequence of amino acids 1–518 of ZUD. The hybridization was performed with the radiolabeled ZUD cDNA probe in rapid hybridization buffer (Clontech, Palo Alto, CA) under high stringency conditions.

Cell Transfection and Reporter Gene Assays—293 cells (2 × 10^6) were seeded on 6-well (35-mm) dishes and transfected the following day by the standard calcium phosphate precipitation method (29). Within the same experiment, each transfection was performed in triplicate, and, where necessary, an empty control plasmid was added to ensure that each transfection received the same amount of total DNA. To normalize for transfection efficiency, 0.3 μg of a Rous sarcoma virus β-galactosidase plasmid was added to each transfection. Luciferase reporter assays were performed using a luciferase assay kit (BD Biosciences, San Diego, CA) under high stringency conditions.

Immunofluorescent Staining—293 cells cultured on glass coverslips were sequentially plunged into methanol at −20°C for 10 min. The cells were rehydrated in PBS, blocked with 1% bovine serum albumin in PBS for 30 min, and stained with a monoclonal anti-FLAG antibody (2 μg/ml) in blocking buffer for 1 h at room temperature. The cells were rinsed with PBS and stained with fluorescein isothiocyanate-conjugated goat anti-mouse IgG (1:200 dilution) for 45 min at room temperature. The cells were then rinsed with PBS containing Hoechst 33342 and mounted in Prolong Antifade (Molecular Probes, Eugene, OR). The cells were observed with a Leica DMR/XA immunofluorescent microscope using a 40× plan objective.

Apoplosis Assays—β-Galactosidase co-transfection assays for determination of cell death were performed as described previously (9–11, 28, 31). Briefly, 293 cells (2 × 10^6) were seeded on 6-well (35-mm) dishes and transfected the following day with 0.1 μg of a cytotoxic receptor mouse IgG and 25/μl of aprotinin, 10/μl of leupeptin, and 1 μl of phenylmethylsulfonyl fluoride. For each immunoprecipitation, a 0.4-μl aliquot of lysate was incubated with 0.5 μg of the indicated monoclonal antibody or control mouse IgG and 25 μl of a 1:1 slurry of GammaBind G Plus-Sepharose (Amersham Biosciences) for at least 1 h. The Sepharose beads were washed three times with 1 ml of lysate buffer containing 500 μl NaCl. The precipitates were fractionated on SDS-PAGE, and subsequent Western blot analyses were performed as described (27–30).

Identification and Cloning of ZUD—To identify a potential DD-containing protein, we searched the GenBank™ EST databases using the tBLASTn program. This search identified multiple human EST clones that encode a novel DD-containing protein, which was designated ZUD. The longest EST clone (GenBank™ accession number BI762014) encodes the full-length human ZUD protein because it contains an in-frame stop codon 5’ of the putative ATG start codon and a poly(A) signal sequence at the 3’-end (data not shown). Sequence analysis indicated that human ZUD cDNA encodes a 518 amino acid protein (Fig. 1A). BLAST searches of the GenBank™ databases indicated that human ZUD shares ~83% sequence identity with its mouse ortholog at the amino acid level. Structural analysis indicated that ZUD contains an N-terminal ZU5 domain (aa108–186) (a functionally unknown domain found in ZO-1 and UNC5H receptors), a middle leucine zipper motif (aa 249–270), and a C-terminal death domain (aa 423–491) (Fig. 1A). ZUD is mostly homologous to the cytoplasmic fragments of the UNC5H receptor family members, which also contain a ZU5 domain and a death domain (21–26). The overall similarity between ZUD and the cytoplasmic domains of UNC5H receptors is ~40%.

Northern blot analysis indicated that ZUD is expressed in

![Fig. 1. Structure and tissue expression of ZUD.](http://www.jbc.org/Downloaded from http://www.jbc.org/)

**EXPERIMENTAL PROCEDURES**

**Reagents**—The ZUD EST clone (Research Genetics, Huntsville, TN), the recombinant human TNF-α, IL-1, and IFN-γ (R & D Systems Inc., Minneapolis, MN), the monoclonal antibodies against FLAG (Sigma), Myc (Santa Cruz Biotechnology, Santa Cruz, CA), hemagglutinin epitopes (Covance, Berkeley, CA), and the goat polyclonal antibody against p65 and the rabbit polyclonal antibody against p50 and IκBα (Santa Cruz Biotechnology, Santa Cruz, CA) were purchased from the indicated manufacturers. Recombinantly soluble TRAIL was produced in our laboratory (27). Human embryonic kidney 293 cells were obtained from the American Type Culture Collection (Manassas, VA).

**Constructions**—Expression plasmids for TNF-R1, TRAF2, TRAF6, IKK-β, p65, p50, and IκBα (Dr. David Goeddel) and the NF-κB (Dr. Gary Johnson) and IRF-1 (Dr. Uli Schindler) luciferase reporter constructs were provided by the indicated investigators. Mammalian expression plasmids for Myc-tagged p105 and its deletion mutants and for hemagglutinin- or FLAG-tagged ZUD and its deletion mutants were constructed by PCR amplification of the corresponding cDNA fragments and subsequent cloning into a cytomegalovirus promoter-based vector. Mammalian expression plasmid for SINK was described previously (28).

**ZUD Is a Novel NF-κB Inhibitor**
pancreas, kidney, and liver as transcripts of \( \sim 3 \) and \( \sim 4 \) kb respectively (Fig. 1B). ZUD mRNA is barely detectable in other examined tissues, including heart, brain, placenta, lung, skeletal muscle, spleen, thymus, prostate, uterus, testis, small intestine, colon, and peripheral blood leukocytes (Fig. 1B). Analysis of available sequences in the GenBank\textsuperscript{TM} databases (cDNAs, ESTs, and genomic sequences) suggests that the more abundant \( \sim 3 \) kb transcript represents the cDNA sequence we obtained. Because this sequence contains a 5' in-frame stop codon and no splicing or poly(A) signal variants were identified by the GenBank\textsuperscript{TM} data base searches, the \( \sim 4 \) kb transcript may represent an alternative splicing form in which additional 5'-untranslated region sequence is included.

ZUD Inhibits NF-\( \kappa \)B-dependent Transcription—Because ZUD contains a DD, we determined whether ZUD has a role in apoptosis. Transient transfection experiments indicated that overexpression of ZUD could not induce apoptosis or inhibit death receptor-induced apoptosis (data not shown). Because many DD-containing proteins are involved in NF-\( \kappa \)B activation and the NF-\( \kappa \)B subunits p105 and p100 themselves contain DDs, we determined whether ZUD has a role in NF-\( \kappa \)B activa-
Fig. 4. ZUD interacts with p105 and p65. A, ZUD interacts with p65. 293 cells \((-2 \times 10^6)\) were transfected with expression plasmids for FLAG-tagged ZUD, together with p65, p50, IκBa, or Myc-tagged IKKα or IKKβ. Cell lysates were immunoprecipitated with control mouse IgG or a monoclonal anti-FLAG antibody (αF). The immunoprecipitates were analyzed by Western blots with goat polyclonal anti-p65, rabbit polyclonal anti-p50 and IκBa, and mouse monoclonal anti-Myc antibodies, respectively. Horseradish peroxidase-conjugated secondary antibodies were used for visualizing the proteins by enhanced chemiluminescence. Expression levels of p65, p50, IκBa, IKKα, and IKKβ were also examined by Western blots (Lysates). B, ZUD interacts with p105 and its C terminus. 293 cells \((-2 \times 10^6)\) were transfected with expression plasmids for FLAG-tagged ZUD, together with Myc-tagged p105 or its deletion mutants p105 (aa 1–434) or p105 (aa 435–969). Cell lysates were immunoprecipitated with control mouse IgG or a monoclonal anti-FLAG antibody (αF). Western blots of the immunoprecipitates (left panel) or lysates (right panel) were performed with a mouse monoclonal anti-Myc antibody directly conjugated with horseradish peroxidase.

**DISCUSSION**

In an attempt to clone novel DD-containing proteins, we isolated ZUD by searching human EST databases. ZUD contained a conserved ZU5 domain, a middle leucine zipper motif, and a C-terminal DD. Analysis of the amino acid sequence of ZUD suggests that it is homologous to the cytoplasmic domains of a group of netrin-1 receptors, UNC5H1–4 (21–26). These receptors were initially proposed as mediators of the chemorepulsive effects of netrin-1 on specific axons (22). Although UNC5H receptors are highly expressed in specific neurons during the development of the nervous system in adults, their expression can also be detected in thyroid, kidney, ovary, uterus, stomach, colon, lung, spleen, bladder, and breast tissues (23). These observations suggest a role for these receptors away from the neuronal guidance system. Recently, it has been shown that these receptors, when unbound to their ligand, induce apoptosis (24). One of the UNC5H genes, UNC5H2/B, was recently shown to be a direct transcriptional target of the tumor suppressor p53 and to mediate the proapoptotic activity of p53 (25). In addition, human UNC5H1, UNC5H2, and UNC5H3 are down-regulated in multiple cancers, and overexpression of these receptors inhibits anchorage-independent growth and invasion of tumor cells (26). Because ZUD is undetectable in the brain, it may have no role in UNC5H-mediated chemo-repulsive effects. However, it is possible that ZUD may be involved in other UNC5H-mediated effects. In transient
transfection and co-immunoprecipitation experiments, we found that ZUD could interact with UNC5H2 in 293 cells (data not shown), pointing to the possibility that they are functionally connected. It has been suggested that UNC5H induces apoptosis when it is not bound with its ligand (24). Our future studies will investigate whether ZUD is involved in this process.

Overexpression of ZUD inhibited TNF- and IL1-

Fig. 5. Domain mapping of ZUD interaction with p105 and p65. A, schematic presentation of ZUD deletion mutants. B, expression of the ZUD deletion mutants. 293 cells (~2 × 10⁶) were transfected with expression plasmids for the indicated FLAG-tagged ZUD mutants. Cell lysates were analyzed by Western blot with anti-FLAG antibody. C, the middle leucine zipper-containing domain of ZUD interacts with p65. 293 cells (~2 × 10⁶) were transfected with expression plasmids for p65 and the indicated FLAG-tagged ZUD mutants. Cell lysates were immunoprecipitated with control mouse IgG or a monoclonal anti-FLAG antibody (αF). The immunoprecipitates were analyzed by Western blot with anti-p65 antibody. D and E, the middle leucine zipper-containing domain of ZUD interacts with p105 and its C terminus. 293 cells (~2 × 10⁶) were transfected with expression plasmids for Myc-tagged p105 (D) or p105 (aa 435–969) (E) and the indicated FLAG-tagged ZUD mutants. Cell lysates were immunoprecipitated with control mouse IgG or a monoclonal anti-FLAG antibody (αF). The immunoprecipitates were analyzed by Western blot with anti-Myc antibody.

overexpression of p65 (Fig. 2). In gel shift assays, overexpression of ZUD inhibited TNF-induced NF-

Because ZUD is primarily a cytoplasmic protein (Fig. 3A), our original hypothesis was that ZUD might act by retaining NF-κB, especially the p65 subunit, in the cytoplasm. However, immunofluorescent staining experiments indicated that overexpression of ZUD did not block TNF-induced translocation of p65 and p50 to the nucleus (data not shown). Currently, the mechanisms responsible for the inhibitory effects of ZUD are unknown. One of the possibilities is that ZUD affects modification of p65 in the cytoplasm, such as phosphorylation, and this leads to inhibition of NF-κB binding to its target sequence and the subsequent inhibition of NF-κB-mediated transcription. In this context, it has been shown that p65 phosphorylation is important for the full transcription competence of NF-κB (37–41). Consistent with its role in inhibiting NF-κB-dependent transcription, ZUD also sensitizes cells to TNF- and TRAIL-induced apoptosis, probably through the inhibition of expression of NF-κB activated anti-apoptotic genes.

Taken together, our findings provide evidence that ZUD is a novel NF-κB inhibitor with functional similarity to IkB proteins. Previously, it has been shown that p65 interacts directly
with multiple proteins other than the IκB family, including p202 (42), CREB-binding protein/p300 (41), Stat 6 (44), TAFII105 (45), RAI (41), SINK (28), AO7 (43), and BRCA1 (33), which either suppress or activate NF-κB. It is possible that these NF-κB-interacting proteins, including ZUD, are involved in a coordinated regulation of its activity in either a tissue-specific or nonspecific manner.

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