SIMPL Is a Tumor Necrosis Factor-specific Regulator of Nuclear Factor-κB Activity*

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The IL-1 receptor-associated kinase (IRAK/mPLK) is linked to the regulation of nuclear factor-κB (NF-κB)-dependent gene expression. Here we describe a novel binding partner of IRAK/mPLK that we term SIMPL (signal diminished. Moreover, dominant-negative SIMPL blocks regulation of NF-κB-dependent signaling. Inactivation of NF-κB protein activity is the result of rapid stimulus-dependent phosphorylation on two critical amino-terminal serine residues (7–10). The phosphorylations are a prerequisite for ubiquitin-targeted proteasome-dependent degradation of IkB. A large multiprotein complex that includes at least the IkB kinases (IKKα and IKKβ) and the scaffolding protein IKKγ (NEMO) has been described previously (11–18). IKKα and IKKβ are responsible for IkB phosphorylation and are themselves substrates of phosphorylation events necessary for IKK activation (for review see Refs. 19 and 20).

The IL-1 receptor-associated kinase (IRAK) was identified as a protein associated with the type I IL-1 receptor (21), whereas the mouse homologue of IRAK, mouse pelle-like kinase (mPLK), was identified independently (22) in a cDNA library screened for kinases related to the Drosophila pelle kinase (23). We have demonstrated that IRAK/mPLK protein kinase activity is critical for TNF-RI signaling and placed mPLK/IRAK in a novel signal transduction pathway through which TNF-RI activates NF-κB-dependent gene expression (24). Although the IRAK/mPLK protein may be required for IL-1-induced NF-κB activity, IRAK/mPLK catalytic activity is not required for IL-1-dependent signaling (24–26). In mPLK/IRAK null fibroblasts IL-1 as well as in TNF-induced NF-κB, DNA binding activity is significantly attenuated (27). In this report we describe a novel signaling molecule that associates with the mouse pelle-like kinase (SIMPL) and demonstrate that SIMPL is required for TNF-RI-dependent activation of NF-κB activity.

**EXPERIMENTAL PROCEDURES**

**Library Screening**—DNA fragments encoding different regions of the IRAK/mPLK protein (21, 24) were subcloned into pGBDU and transformed into the yeast strain PJ69-4A (28). Transformants were selected on Sd/-Ura, Sd/-Ade, Sd/-His for test for autoactivation of the reporter genes. A pGBDU construct encoding amino acids 1–533 of mPLK (pGBDU-mPLK534–710) that did not activate transcription in the absence of a bait plasmid was subjected to a two-hybrid analysis. A day 17 mouse embryo Matchmaker cDNA library (CLONTECH, Palo Alto, CA) was transformed into PJ69-4A expressing pGBDU-mPLK534–710. Seven million transformants were screened for β-galactosidase activity. Transformants were plated onto Sd/-Ura/-Leu/-His, 4 mit 3-aminothiol plates. 4–9 days later transformants were streaked onto Sd/-Ura/-Leu/-Ade plates. Replating was repeated until all remaining colonies could grow on Sd/-Ura/-Leu/-His, 4 mit 3-aminothiol, and Sd/-Ura/-Leu/-Ade plates. β-Galactosidase activity was tested by the standard 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal) filter lift assay. β-Galactosidase-positive clones were grown on Sd/-Leu + 5-fluoro-otic acid plates to force loss of the bait plasmid pGBDU-mPLK534–710. All positive constructs were tested for trap-independent activation by growth on Sd/-Leu/-Ade and Sd/-Leu/-His, 4 mit 3-aminothiol plates. Plasmids purified from PF69-4A transformants were transformed into the bacterial strains DH5α or RR1 by electroporation. Bacterial transformants con-
taining the pGAD10 vector constructs were selected on M9-/Leu plates. Positive clones were retransformed with yeast strain PJ69-4A containing pGBDU-mPLKΔ534–710 with the positive pGAD10 construct and selecting for growth on SD/-Ura-/Leu-His and -tryptophan Leu-Ade plates. Inserts were excised from the pGAD10 vector with the EcoRI restriction endonuclease and subcloned into a mammalian expression vector (pcDNA3.0 or pcDNA3.1, Invitrogen, Carlsbad, CA). Nucleotide sequence analysis was determined on both strands by the University of California Biotechnology Facility with an Applied Biosystems Inc. automated sequencer.

**Northern Blot Analysis—**Northern blots containing mRNA extracted from developing mouse embryos and a panel of adult tissues (CLONTECH) were probed with a SIMPL cDNA radiolabeled by random priming [32P]dCTP (3000 Ci/mmol) (Amersham Pharmacia Biotech) according to the manufacturer's recommendations.

**Plasmid Constructs and Antibodies—**The IL-8-Luc and (NF-κB)-Luc reporter constructs and IRAK/mPLK expression constructs have been described previously (24). The SIMPL cDNA was subcloned into a mammalian expression vector that placed the SIMPL coding region under the control of the cytomegalovirus immediate early gene promoter (pFLAG-CMV2, Eastman Kodak). The wild-type and catalytically inactive versions of IKKα and IKKβ (15) were kindly provided by Michael Karin (University of California, San Diego). The mouse e-cmyc monoclonal antibody 9E10 was purchased from Roche Molecular Biochemicals. A peptide fragment corresponding to amino acids 200–213 of SIMPL conjugated to keyhole limpet hemocyanin was used to immunize a female chicken (Gallus Immunotech, Ontario, Canada). Crude egg lysates were enriched for IgY-containing protein by affinity chromatography. In pilot studies, we determined that the SIMPL antiserum could be used for Western blot analysis but was not suitable for immunocomplexing assays (data not shown).

**Cell Culture and Transfections—**Human embryonic kidney 293 (HEK 293) epithelial cells and C3H10T1/2 mouse embryo fibroblast cell lines were maintained and transfected as described previously (24). To monitor transfection efficiencies, precipitates also included a construct containing the Renilla luciferase cDNA. Cultures were harvested 24 or 48 h following transfection, and luciferase activities were determined using the Dual-Luciferase® reporter assay system (Promega, Madison, WI) according to the manufacturer's specifications. Individual assays were normalized for Renilla luciferase activity, and data are presented as the fold increase in activity relative to empty vector control. Data are from 2 to 3 independent experiments performed in duplicate or triplicate with similar qualitative results with standard errors indicated.

**TUNEL Assays—**HEK 293 cells (2 × 10⁵) were transfected with a mammalian expression vector encoding ΔSIMPL or ΔSIMPL plus a mammalian expression vector encoding CrmA. To control for the presence of nonapoptotic cells, one set of cultures was transfected with the vector. 24 h later cells were harvested, fixed, and permeabilized as described below. As a positive control, fixed permeabilized cells were treated with DNase I prior to analysis. To detect double strand DNA breaks, terminal deoxynucleotidyltransferase was used to add fluorescein-labeled nucleotides to free 3'-breaks in the DNA strands with an in situ cell death detection kit (Roche Molecular Biochemicals). Flow cytometry was used to detect cells containing fluorescein-labeled DNA.

**Immunoprecipitations and Western Blotting—**Immunoprecipitations, SDS-polyacrylamide gel electrophoresis, and Western blot analysis were performed as described previously (24).

**IRAK/mPLK Nulligenic ES Cells—**IRAK/mPLK nulligenic ES cells were generated with the pPLK/IRAK-targeting construct as described by Thomas et al. (27). The 129SV-derived ES cell line R1 was maintained in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum, 100 IU/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamine, 1.0 mM sodium pyruvate, 0.1 mM 2-mercaptoethanol, and 1.2 × 10⁶ units/ml leukemia inhibitory factor (LIF, Life Technologies, Inc.) on monolayers of mitomycin C-treated STO cells (subline of SIM mouse fibroblasts). To introduce the targeting vector, 8 × 10⁶ R1-ES cells were seeded in 0.5 ml of medium containing 40 μg/ml of the linearized targeting construct. Electroporated cells were incubated on ice for 20 min before co-culturing with the mitomycin-treated STO monolayers. Three days after electroporation, cell cultures were treated with 400 μg/ml G418 (Life Technologies, Inc.) and 2 μg gancyclovir. Individual colonies were isolated 10 days later. Genomic DNA was purified from G418 and gancyclovir-resistant ES cell colonies with a DNA isolation kit (Puregene, Minneapolis, MN). The first round of screening was performed by polymerase chain reaction, and positives were re-screened by Southern blot analysis. Genomic DNA was digested with the BamHI restriction endonuclease and separated on a 0.8% agarose gel in 1× a buffer containing 40 mM Tris acetate, 1 mM EDTA. The DNA was transferred to a nylon membrane (MSI, Westboro, MA) and probed with genomic fragments that distinguish the wild-type and targeted allele using IRAK/mPLK genomic probes outside the region that is used to generate the targeting construct. Only one targeting event was necessary as the mPLK gene is located on the X chromosome.

**Liquid Chromatography Electrospray Mass Spectrometry—**Immunocomplexed materials were subject to SDS-polyacrylamide gel electrophoresis, and individual protein bands were excised and digested with trypsin. Solubilized samples were analyzed by capillary liquid chromatography using an ABI 140D solvent delivery system. Samples were applied directly to 300-μm inside diameter fused silica capillaries packed with Vydac C18 resin and separated with gradients of Buffer A (2% acetonitril and 98% H₂O containing 0.2% (propyol alcohol, 0.1% acetic acid, and 0.001% trifluoroacetic acid). Peptides were eluted with a flow rate of 7 μl/min directly into the electrospray ionization source of a Finnigan LCQ mass spectrometer. Nitrogen was used as the sheath gas with a pressure of 35 p.s.i., and no auxiliary gas was used. Electrospray ionization was conducted with a spray voltage of 4.8 kV, a capillary temperature of 200 °C, and a capillary voltage of 20 V. Spectra were scanned over a range of 200-2000 m/z. Base peak ions were trapped using a quadrupole ion trap and further analyzed with a high resolution zoom scan using an isolation width of 3 m/z and collision-induced dissociation scans with a collision energy of 40.
mPLK protein was used to generate immunocomplexes from HEK 293 cells. As a negative control, immunocomplexes were also generated with a nonspecific mouse IgG. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis, and Western blots were prepared and probed with antiserum to IRAK/mPLK or antiserum to SIMPL. SIMPL protein is detected in immunocomplexes generated with the IRAK/mPLK antisera (Fig. 1D, lane under aIRAK). Neither IRAK/mPLK nor SIMPL is found in immunocomplexes generated with an unrelated mouse IgG (Fig. 1D, lane under IgG). Detection of IRAK/mPLK in immunocomplexes with SIMPL suggests that the interaction initially detected in the yeast two-hybrid screen can be detected in intact cells.

In an independent experiment, a SIMPL-derived peptide was identified (Fig. 1A, underscored residues) in Myc-tagged IRAK/mPLK immunocomplexes generated with an unrelated mouse IgG (Fig. 1D, lane under IgG). Detection of IRAK/mPLK in immunocomplexes with SIMPL suggests that the interaction initially detected in the yeast two-hybrid screen can be detected in intact cells.

Transactivation of NF-κB-dependent Gene Expression by SIMPL—NF-κB is a key regulator of the immune and stress responses in mammals, and NF-κB activity is increased in response to a variety of stimuli (for review see Ref. 1). Thus, we examined whether SIMPL, like IRAK/mPLK, induces NF-κB activity (24). In these experiments, two different reporter constructs were used: a luciferase cDNA under the control of NF-κB-dependent IL-8 gene promoter (IL-8-Luc) and a luciferase cDNA under the control of three tandem NF-κB sites ((NF-κB)3-Luc). Transient transfection of a mouse embryo fibroblast cell line with a SIMPL cDNA results in a dose-dependent induction of IL-8-Luc and (NF-κB)3-Luc activity (Fig. 2, A and B) with no effect on an activating protein-1-dependent reporter construct (data not presented). Therefore, like IRAK/mPLK, SIMPL lies in a signaling pathway upstream of NF-κB.

With the goal of generating a SIMPL mutant that could be used to study the role of SIMPL in IRAK/mPLK-dependent signaling, a SIMPL mutant (ΔSIMPL), in which the first 80 amino acid residues are missing, was generated. In preliminary studies we noted that the expression of ΔSIMPL decreased cell survival. Thus, we examined directly whether ΔSIMPL expression was pro-apoptotic. HEK cells were transiently transfected with an empty vector or ΔSIMPL in the absence or presence of CrmA, a nonspecific caspase inhibitor from the cowpox virus (29). 24 h later, cultures were harvested.
and analyzed for the presence of DNA double strand breaks (in situ cell death detection kit, Roche Molecular Biochemicals). Fluorescence-activated cell sorting analysis revealed that the expression of SIMPL leads to an increase in the number of cells containing double-stranded DNA breaks as measured by an increase in fluorescence, which are not detected in the presence of CrmA (Fig. 2C). Based on these results, in subsequent experiments in which the ΔSIMPL mutant was analyzed CrmA was also included. To determine whether the ΔSIMPL mutant functioned as a dominant-negative, wild-type SIMPL was coexpressed with increasing amounts of ΔSIMPL. Expression of ΔSIMPL decreases in a dose-dependent manner, SIMPL-induced NF-κB activity (Fig. 2D). Taken together, these results reveal that ΔSIMPL functions as a dominant-negative allele of SIMPL, and SIMPL, like NF-κB, appears to be critical for cell survival.

To define the functional relationship between IRAK/mPLK and SIMPL, we examined whether the ability of SIMPL to induce NF-κB activity was IRAK/mPLK-dependent. For these studies, a clone of embryonic stem cells nulligenic for mPLK (ES −/−, see “Experimental Procedures”) was analyzed and compared with wild-type ES cells (ES +/+). Overexpression of SIMPL in ES +/+ cells increases IL-8-Luc promoter activity 3-fold (Fig. 3A). In contrast, overexpression of SIMPL in the ES −/− cells does not increase IL-8-Luc promoter activity (Fig. 3A). Thus, the ability of SIMPL to induce NF-κB activity is dependent upon the presence of the IRAK/mPLK protein. We next determined whether IRAK/mPLK-induced NF-κB activation is SIMPL-dependent by examining whether ΔSIMPL blocked IRAK/mPLK-induced NF-κB activity. Coexpression of ΔSIMPL with IRAK/mPLK inhibits IRAK/mPLK activity (Fig. 3B). Taken together, these data support the hypothesis that SIMPL is a component of an IRAK/mPLK-dependent pathway that controls NF-κB activity.

Our group has identified a requirement for IRAK/mPLK catalytic activity in TNF-RI-dependent induction of NF-κB ac-

![Fig. 2. SIMPL-induced NF-κB activity. Mouse embryo fibroblasts were transiently transfected with an IL-8-Luc reporter construct (A) or a (NF-κB)-3-Luc reporter construct (B) and constructs encoding the Renilla luciferase cDNA and the indicated amounts of an eukaryotic expression vector containing no insert (pCMV), the same eukaryotic expression vector containing the SIMPL cDNA and/or the ΔSIMPL cDNA. C, HEK 293T cells were transfected with the indicated constructs; 24 h later cultures were harvested, and TUNEL assays were performed as described under “Experimental Procedures.” The y axis indicates the cell number, and the x axis indicates the fluorescence intensity. The gray curve present in each histogram depicts results obtained with nontransfected control cells. D, mouse embryo fibroblasts were transiently transfected with an IL-8-Luc reporter construct, the Renilla luciferase cDNA, CrmA, and the indicated SIMPL constructs. For experiments presented in A, B, and D, the error bars are mean ± S.D. of duplicate samples. All experiments were repeated three times.](image-url)
tivity that occurs in a TRADD-independent manner (24). Therefore, it was of great interest to determine whether SIMPL, like IRAK/mPLK, is required for TNF-RI-induced NF-κB activity. Consistent with the hypothesis that SIMPL is downstream of IRAK/mPLK, D SIMPL inhibits TNF-RI-induced NF-κB activity (Fig. 3C). To determine whether ΔSIMPL-induced inhibition is specific for TNF-RI, the effect of ΔSIMPL on IL-1-induced NF-κB activity was measured. Expression of ΔSIMPL does not inhibit IL-1-induced NF-κB activity (Fig. 3D). In parallel to the effect seen when catalytically inactive IRAK/mPLK is expressed (24–26), ΔSIMPL appears to enhance IL-1-dependent induction of NF-κB activity. Thus, like IRAK mPLK catalytic activity, SIMPL is also required for TNF-RI-dependent induction of NF-κB activity.

IKKα and IKKβ Mutants Inhibit IRAK/mPLK- and SIMPL-induced NF-κB Activity—Current models predict that IKKα and IKKβ are required for the activation of NF-κB-dependent gene expression (30–33). Thus, we examined whether SIMPL-induced NF-κB activation requires IKKα and/or IKKβ activity. Substitution of the lysine residue at position 44 for methionine within IKKα (IKKαKM) and for alanine within IKKβ (IKKβKA) results in the loss of IKK catalytic activity (15). IKKαKM or IKKβKA attenuate IRAK/mPLK-induced NF-κB activity and SIMPL-induced NF-κB activation (Fig. 3A and 3B, respectively). Thus, like TNF-RI (30–33), IKKα and IKKβ are necessary components of the pathway through which IRAK/mPLK and SIMPL signal for activation of NF-κB. We next examined whether IKK-induced NF-κB activity is affected by ΔSIMPL expression. ΔSIMPL inhibited IKKα- and IKKβ-induced NF-κB activity (Fig. 3C). These data suggest a model in which SIMPL integrates the activities of upstream activators, like IRAK/mPLK, with the IKK-containing complex(es).

To evaluate the role of the SIMPL protein in physical interactions between IRAK/mPLK and the IKKs, we first examined whether expression of a SIMPL antisense construct would decrease steady-state levels of SIMPL protein and protein activity. Mouse embryonic fibroblasts were transfected with the IL-8 luciferase and Renilla luciferase reporter constructs, a mammalian expression vector encoding an epitope-tagged version of SIMPL (SIMPL-FLAG) and an increasing amount of a SIMPL antisense construct. Because the dominant-negative allele of SIMPL induced an apoptotic response, a mammalian expression vector encoding CrmA was included with the transfected cell cultures.
DNAs. In the presence of the SIMPL antisense construct there is a dose-dependent decrease in SIMPL-induced NF-κB activity (Fig. 5A) and a decrease in the steady-state levels of SIMPL protein (Fig. 5B). We also examined whether transfection with the SIMPL antisense construct would result in a decrease in the steady-state level of endogenous SIMPL protein. Consistent with the results obtained with the FLAG-tagged SIMPL construct, transfection of the SIMPL antisense construct leads to a decrease in the steady-state level of endogenous SIMPL protein (Fig. 5C). Thus, the transfection of HEK cells with the SIMPL antisense construct results in decreased SIMPL protein and protein activity.

The data presented thus far demonstrate that the SIMPL and IKK protein activities are interdependent. Consequently, we were interested in determining whether IRAK/mPLK and SIMPL proteins could be found in IKK-containing complexes.

We first examined whether IRAK/mPLK-SIMPL could be found in IKKα- and/or IKKβ-containing complexes. To test this hypothesis, IRAK/mPLK antisera or a mouse IgG control was used to generate immunocomplexes that were subjected to SDS-polyacrylamide gel electrophoresis followed by Western blotting. Analysis of the Western blot probed with the SIMPL antiserum revealed the presence of SIMPL in complexes obtained with the IRAK antisera (Fig. 5D, middle lane) but not in complexes generated with a mouse IgG control (Fig. 5D, first lane). When the Western blot containing the immunocomplexes that were generated with the IRAK/mPLK antiserum was probed with antisera to IKKα or IKKβ, both proteins were detected (Fig. 5D, middle lane). Based on these data, we hypothesize that complexes containing IRAK/mPLK, SIMPL, IKKα, and IKKβ can be isolated from cells under steady-state conditions.

To determine whether there is a requirement for SIMPL in IRAK/mPLK/IKKα/IKKβ complex formation, we examined whether the SIMPL antisense construct would affect IRAK/mPLK/IKKα/IKKβ complex formation. In these experiments the SIMPL antisense construct was introduced into HEK 293 cells, and immunocomplexes were generated with antibody to endogenous IRAK/mPLK protein. Western blots were prepared and probed with SIMPL, IRAK/mPLK, IKKβ, and IKKα antisera. In the IRAK/mPLK-containing immunocomplexes isolated from cultures expressing the antisense SIMPL construct, neither the SIMPL protein nor the IKKβ proteins were detected in association with IRAK/mPLK (Fig. 5D, last lane). Intriguingly, the IKKα protein was detected in the IRAK/mPLK protein complexes independent of SIMPL.

In summary, SIMPL is a novel component of the IRAK/mPLK-dependent TNF-RI signaling pathway that leads to the activation of NF-κB. Several different sets of data support a link
of wild-type SIMPL leads to induction of the NF-κB activity, which is associated with cell survival, and high levels of SIMPL transcripts are detected in the brain and testis, immune-privileged tissues in which cell survival is paramount. Recently, Pfeuffer et al. (36) isolated a truncated version of SIMPL (amino acids 52–259) in a two-hybrid screen for proteins that bind ActA, a critical factor in the pathogenesis of a Listeria monocytogenes infection. Intriguingly, unlike wild-type animals, a L. monocytogenes infection in TNF−/− or TNF-RI−/− animals is lethal (37, 38).

Several groups of investigators including ourselves have demonstrated that mPLK/IRAK catalytic activity is not required for IL-1 induction of an NF-κB-dependent response (24–26). Our group has identified a requirement for mPLK/IRAK catalytic activity in TNF-RI-dependent induction of NF-κB activity that occurs in a TRADD-independent manner (24). Because dominant-negative SIMPL blocks a TNF-RI but not an IL-1RI response, our data support a model in which SIMPL is a component of the mPLK/IRAK-dependent signaling pathway that requires IRAK/mPLK catalytic activity and lies downstream of TNF-RI.

The precise function of SIMPL is unclear. SIMPL appears to facilitate and/or regulate complex formation between IRAK/ mPLK- and IKK-containing complexes. The evidence presented herein support the existence of a TNF-RI-dependent IRAK/ mPLK-SIMPL-IKKβ-dependent signaling pathway. Tujima et al. (39) recently reported that NF-κB-activating kinase couples protein kinase C activity to IKKα/β-containing complexes. These data combined with the results presented herein and elsewhere (for review see Ref. 40) suggest that ligand-dependent NF-κB activation events that occur coordinate with 1B protein phosphorylation and degradation converge at the level of the IKKs.

Acknowledgments—We thank M. Karin for supplying us with IKK constructs and J. Hawes (Indiana University School of Medicine, Indianapolis, IN) for the ionizing mass spectroscopy analysis.

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