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J Immunol (2002) 169 (8): 4075–4078.

<https://doi.org/10.4049/jimmunol.169.8.4075>

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Cutting Edge: Mouse Pellino-2 Modulates IL-1 and Lipopolysaccharide Signaling¹

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Pellino is a *Drosophila* protein originally isolated in a two-hybrid screen for proteins interacting with the serine/threonine kinase, pelle. Although mammalian homologs have been identified in mouse and man, the function of pellino is as yet unknown. In this study, the cloning, expression pattern, and a preliminary characterization of mouse pellino-2 is described. These studies reveal that mouse pellino-2 is expressed during embryogenesis and in a tissue-restricted manner in the adult. IL-1 induces the association of mouse pellino-2 with the mouse pelle-like kinase/IL-1R-associated kinase protein, a mammalian homolog of pelle. Ectopic pellino-2 expression did not result in NF- κ B activation. However, ectopic expression of a mouse pellino-2 antisense construct inhibited IL-1 or LPS-induced activation of NF- κ B-dependent IL-8 promoter activity. Our data reveal that mouse pellino-2 is a tissue-restricted component of a signaling pathway that couples the mouse pelle-like kinase/IL-1R-associated kinase protein to IL-1- or LPS-dependent signaling. *The Journal of Immunology*, 2002, 169: 4075–4078.

The innate immune response is rapidly activated upon exposure to environmental stimuli and discriminates between self and nonself. *Drosophila*, like other invertebrates, relies entirely on innate mechanisms for host defense. Toll receptor activation in *Drosophila* and in mammals leads to changes in Rel-dependent gene transcription. The functional similarity and sequence conservation shared between the *Drosophila* Toll receptor, the related mammalian Toll-like receptors (TLRs),³ and type I IL-1R cytoplasmic domains suggests their respective downstream signaling pathways may contain homologs in addition to those currently known (1, 2).

Pelle is a serine/threonine kinase that is a component of the *Drosophila* Toll signaling pathway which controls dorsal/ventral polarity in the early embryo and is required for protection against fungal infections in the adult (3). Four mammalian pelle homologs have been described: the mouse pelle-like kinase (mPLK)/IL-1R-associated kinase (IRAK)-1, IRAK-2, IRAK-M, and IRAK-4, and each has been linked to Toll as well as IL-1R signaling (4–7). Current models predict that in response to IL-1 binding the type I IL-1R, the IL-1R accessory protein and MyD88 are recruited to the receptor. The IL-1R accessory protein and/or MyD88 bind mPLK/IRAK-1 (8, 9) and TNFR-associated factor-6. Through an unknown mechanism, MyD88-mPLK/IRAK-1-TNFR-associated factor-6 activate the I κ B kinase complex, which in turn phosphorylates I κ B α thereby allowing for activation of Rel (NF- κ B)-dependent gene expression (5, 10, 11). LPS binding to TLR4 is thought to stimulate activation of NF- κ B-dependent transcription in a similar manner (for review see Ref. 12). The precise role of mPLK/IRAK-1 in IL-1 and/or Toll-dependent activation of NF- κ B-dependent gene transcription is unclear; in these particular signaling pathways, mPLK/IRAK-1 protein, but not its cognate catalytic activity, is required (13).

Although a primarily biochemical approach has provided insight into downstream components of the IL-1 type I receptor and TLR signaling pathways, genetic studies have defined several components of the *Drosophila* Toll signaling pathway. One of these components is tube, an adapter protein that together with pelle is required for cactus phosphorylation and nuclear relocalization of the Rel family member, dorsal (14, 15). To gain insight into the requirement for pelle in cactus/dorsal complex activation, Grosshans et al. (16) conducted a two-hybrid screen with pelle that resulted in the identification of pellino. Grosshans et al. determined that pellino associates with the kinase domain of pelle; however, a role and/or requirement for pellino in the Toll pathway was not reported. Database searches revealed that pellino is an evolutionarily conserved protein (17, 18). Based on the presumptive identification of pellino homologs in the mouse database, we examined whether a mouse pellino homolog may be involved in modulating mPLK/IRAK-1 activity.

Materials and Methods

Isolation and cloning of mouse pellino-2

Primers were designed (5'-TCTCTAGGTTTCGCATGCAGGATCGTGTG-3' and 5'-ACTGGGGTCGGGCTGCATTGATCTCCTG-3') to RT-PCR partial pellino-2 sequence from total RNA isolated from mouse embryonic fibroblasts (C3H10T1/2 cell line). Nucleotide sequence was determined by the Indiana University Biotechnology Facility (Indianapolis, IN) with an Applied Biosystems (Foster City, CA) automated sequencer. Using this sequence, primers were designed (5'-GCTTTCTGTTGACCTGCCACCTG-3' and 5'-TGCCCTCTATAAGCGGACCTACGCC-3') to

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Received for publication May 15, 2002. Accepted for publication August 14, 2002.

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¹ This research was supported in part by the National Institutes of Health Grant AI42798 (to M.A.H.).

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³ Abbreviations used in this paper: TLR, Toll-like receptor; mPLK, mouse pelle-like kinase; IRAK, human IL-1R-associated kinase; HA, hemagglutinin; LUC, luciferase; HEK, human embryonic kidney.

isolate the full-length mouse pellino sequence, by RACE with the Marathon cDNA Amplification kit (Clontech Laboratories, Palo Alto, CA) according to manufacturer's recommendations. Full-length mouse pellino-2 cDNA was cloned into pCR2.1 vector (Invitrogen, San Diego, CA) and subsequently subcloned into a hemagglutinin (HA)-tagged mammalian expression vector, pHA-CMV (Clontech Laboratories) with the restriction endonucleases *Bam*HI and *Eco*RI. The pellino-2 antisense construct was subcloned into pcDNA3.1 (Invitrogen).

Northern blot analysis

Mouse embryo and multiple tissue blots were purchased from Clontech Laboratories. The mouse pellino-2 cDNA probe was radiolabeled with [α - 32 P]dCTP using a random primer labeling kit (Amersham Pharmacia Biotech, Piscataway, NJ). Hybridization and prehybridization were conducted at 42°C following the manufacturer's recommended procedures.

Cell culture and in vitro assays

The human embryonic kidney (HEK) cells (293-EBNA) and the C3H10T1/2 mouse embryo fibroblast cell line were maintained as described previously (19). Transfections were performed with the indicated DNAs using FuGENE-6 (Roche, Basel, Switzerland) according to the manufacturer's recommendations. Luciferase (LUC) activities were quantitated using the dual luciferase assay system (Promega, Madison, WI).

RT-PCR analysis

Total RNA was isolated using TRIzol (Life Technologies, Grand Island, NY), converted to cDNA by reverse transcription and amplified for 30 cycles by PCR using primers from outside of the antisense region. PCR products were analyzed on agarose gel stained with ethidium bromide and visualized by UV illumination.

Immunoprecipitation and Western blot analysis

The immunocomplexing assays and Western analysis were performed as described previously (19).

Results and Discussion

Before the identification of at least two members of the pellino family homologs in human and mouse (18), a potential mouse pellino homolog was identified by the presence of sequences in the mouse expressed sequence tag database similar to *Drosophila* pellino. Using RT-PCR and RACE, a full-length mouse pellino homolog was generated and isolated from a mouse embryonic fibroblast cell line (C3H10T1/2). The 2-kb insert encodes an open reading frame of 419 aa, and the sequence corresponds to the protein now recognized as mouse pellino-2. Readily discernible protein motifs were not identified in mouse pellino using several different algorithms. Clustal W analysis revealed that mouse pellino-2 and *Drosophila* pellino share 53% similarity, while mouse pellino-1 and pellino-2 share 75% similarity (18).

The endogenous pattern of mouse pellino-2 gene expression was determined by Northern analysis. Two different mRNA species, a 2- and a 6-kb transcript were detected in all tissues examined (Fig. 1). The size of smaller mRNA species matches the size of the cDNA isolated by RACE-PCR; whether the 6 kb corresponds to an alternatively spliced form of pellino-2 is currently unknown. Pellino-2 transcripts were readily detected in the developing mouse embryo. In the adult mouse, pellino-2 transcripts were detected predominantly in the liver, testes, and skin with only low amounts in other tissues. Intriguingly, pellino-2 transcripts were either not detected (spleen) or were barely detectable (thymus) in organs that predominate in the adaptive immune response, but were more abundant in organs (liver and skin) that predominate in the innate immune response.

Drosophila pellino was originally isolated as a pelle-binding protein, thus we examined whether an interaction between mPLK/IRAK-1 and pellino could be detected in mammalian cells. An epitope-tagged version of mouse pellino-2 was generated (HA-Pln-2) and was expressed either alone or with myc-tagged mPLK/IRAK-1 in an HEK cell line (HEK 293-EBNA cells). Western

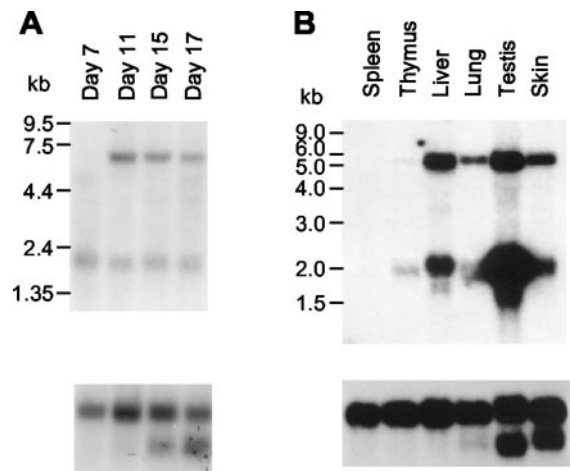


FIGURE 1. Pellino-2 expression pattern. Pellino-2 mRNA expression in mouse embryonic tissues (A) and in adult mouse tissues (B). Blots were probed with pellino-2 cDNA (upper panels) and re-probed with β -actin cDNA (lower panels) to control for loading differences. Molecular size markers are indicated to the left of A and B.

analysis revealed the presence of HA-Pln-2 in immunocomplexes generated with the myc antisera (Fig. 2A) which suggests the mPLK/IRAK-1 and Pln-2 proteins interact in mammalian cells. Another mPLK/IRAK-1-interacting protein is SIMPL, which is required for TNF- α -dependent activation of NF- κ B-dependent gene expression (19). A search of the *Drosophila* database (Fly-Base) failed to identify a *Drosophila* SIMPL homolog, so it was of interest to determine whether pellino-2 and SIMPL interact and form complexes. However, immunocomplexes containing FLAG-tagged SIMPL did not contain pellino-2 (Fig. 2A) suggesting that these proteins do not interact. Taken together, these data reveal that pellino-2 can be detected in mPLK/IRAK-1-containing complexes and the interaction between the two proteins appears specific, as pellino-2 was not detected in immunocomplexes containing another mPLK/IRAK-1-binding protein. To determine whether the interaction between mPLK/IRAK-1 and pellino-2 is modulated in response to IL-1, HEK 293-EBNA cells were transfected with a construct encoding HA-Pln-2. Cell cultures were then treated with

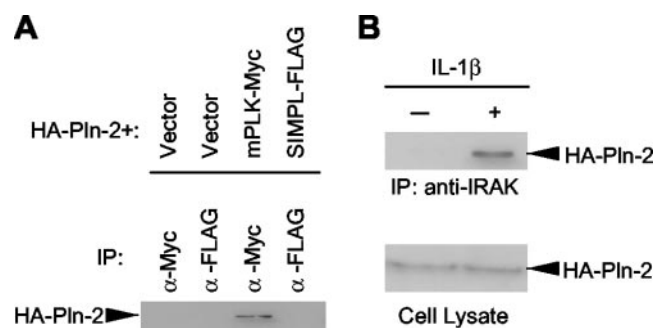


FIGURE 2. IL-1 modulation of pellino-2 and mPLK/IRAK-1 complex formation. A, HEK 293-EBNA cells were cotransfected as previously described (13) with HA-Pln-2 and expression vectors encoding Myc-mPLK/IRAK-1 or FLAG-SIMPL. Anti-Myc or anti-FLAG immunoprecipitates were analyzed by Western blot analysis using anti-HA Ab to detect co-precipitating proteins. B, IL-1 β stimulates the interaction between pellino-2 and mPLK/IRAK-1. HEK 293-EBNA cells transfected with HA-tagged pellino-2 were treated with IL-1 β (1 ng/ml) for 20 min, endogenous mPLK/IRAK-1 was immunoprecipitated with anti-IRAK Ab and Western analysis of the immunocomplexes was performed with anti-HA antisera.

IL-1 β for 20 min before harvest of cell lysates and immunocomplexes generated with antisera that recognize endogenous mPLK/IRAK-1. Western analysis of the mPLK/IRAK-1-containing immunocomplexes revealed that, under steady-state conditions, mPLK/IRAK-1 and pellino-2 are not complexed but mPLK/IRAK-1 and pellino-2 complexes are generated in response to IL-1 stimulation (Fig. 2B). These data suggest that mPLK/IRAK-1-pellino-2 complex formation may be part of an IL-1 signaling pathway.

To investigate the role of pellino-2 in the IL-1 signaling pathway, the ability of pellino-2 to modulate NF- κ B-dependent gene expression was examined. Ectopic expression of pellino-2 in mouse embryonic fibroblasts did not result in activation of a heterologous reporter composed of the firefly LUC cDNA under the control of the NF- κ B-dependent IL-8 gene promoter (data not shown). In contrast to pellino-2, ectopic expression of either mPLK/IRAK-1 or SIMPL leads to a modest increase in IL-8 promoter activity (13, 19). An analysis of a mPLK/IRAK-1 nulligenic demonstrated a requirement for mPLK/IRAK-1 in TNF- α and IL-1-dependent NF- κ B DNA binding activity (20). Work in our laboratory showed that the catalytic activity of mPLK/IRAK-1, as well as presence of SIMPL protein, is required for TNF- α , but not IL-1-, dependent activation of NF- κ B-dependent gene expression (13, 19). Therefore, we decided to examine whether pellino-2 activity would be required for TNF- α and/or IL-1-dependent gene expression. Consistent with results obtained with mPLK/IRAK-1 and SIMPL (13, 19), ectopic expression of pellino-2 had no effect upon IL-8 promoter activity in cultures treated with IL-1, LPS, or TNF- α (data not shown).

The lack of known protein motifs meant that mutagenesis could not be used to investigate a requirement for pellino-2 in IL-1-, LPS-, or TNF- α -dependent activation of NF- κ B activity. Therefore, an antisense approach was used (21, 22). Previous studies in our laboratory have demonstrated that ectopic expression of a cDNA subcloned in the reverse orientation into a mammalian ex-

pression vector will result in a significant drop in the steady-state level of the protein of interest (19). Therefore, pellino-2 cDNA was subcloned in the reverse orientation into pcDNA3.1. To determine the influence of pellino-2 antisense expression, endogenous levels of pellino-2 transcripts were measured, as antisera to endogenous pellino-2 are not currently available. Mouse embryonic fibroblasts were transfected with the pellino-2 antisense construct and endogenous pellino-2 mRNA levels were examined with RT-PCR using primers from 5' and 3' UTR regions which are present in the pellino-2 mRNA but not present in the pellino-2 antisense construct (Fig. 3A). A PCR product for pellino-2 was detected in fibroblasts transfected with the mammalian expression vector that did not contain the pellino-2 antisense construct but this PCR product was dramatically reduced in fibroblasts transfected with the pellino-2 antisense construct (Fig. 3B). Amplification of a PCR product using primers specific to pellino-1 was not affected by expression of the antisense construct (Fig. 3B), confirming the specificity of the pellino-2 antisense construct.

The effect of pellino-2 antisense expression on IL-1, LPS, or TNF- α induction of IL-8 promoter activity was examined. Ectopic expression of the pellino-2 antisense construct inhibited IL-1- or LPS-dependent but not TNF- α -dependent activation of IL-8 promoter activity (Fig. 3C), suggesting a role for pellino-2 in IL-1- or LPS-dependent induction of a NF- κ B-dependent promoter. Several elements important for regulation of the IL-8 gene promoter have been identified including NF- κ B, AP-1, and C/EBP β elements (23). To determine whether the requirement for pellino-2 could be linked to a specific *cis*-acting element, activation of IL-8 reporter constructs containing mutations in the various *cis*-acting elements was examined. Ectopic expression of the pellino-2 antisense construct inhibited IL-1- or LPS-, but not TNF- α -, dependent expression of the IL-8 promoter construct containing the mutated AP-1 element (Fig. 3D). Mutations of either the NF- κ B and/or the C/EBP β elements resulted in a loss of IL-1 or TNF- α inducibility (Fig. 3, E and F) as has been observed by others (23,

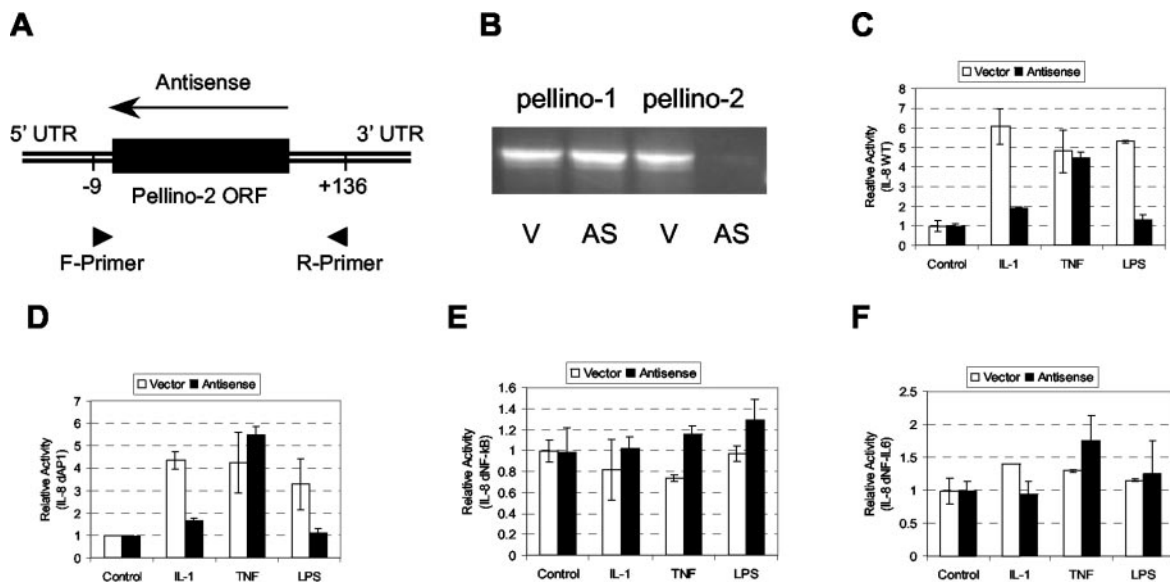


FIGURE 3. Antisense pellino-2 blocks IL-1 or LPS, but not TNF- α , induced IL-8 promoter activity. *A*, Schematic of pellino-2 antisense construct indicating location of primers used for RT-PCR to detect the level of endogenous pellino-2 transcripts. *B*, RT-PCR was performed on mRNA isolated from empty vector (V) or pellino-2 antisense (AS)-transfected C3H10T1/2 cells. *C–F*, Pellino-2 antisense blocks IL-1- or LPS-dependent IL-8 promoter transactivation. C3H10T1/2 cells were cotransfected with empty vector or the pellino-2 antisense construct and the IL-8-LUC and sea pansy LUC constructs (*C*), an AP-1 element deleted IL-8 mutant (IL-8 dAP-1) LUC reporter construct (*D*), a NF- κ B element deleted IL-8 mutant (IL-8 dNF- κ B) LUC reporter construct (*E*), or a NF-IL-6 element deleted IL-8 mutant (IL-8 dNF-IL-6) LUC reporter construct (*F*). After 24 h, cultures were treated with IL-1 β (1 ng/ml), TNF- α (20 ng/ml), or LPS (1 μ g/ml) for 5 h. Cultures were then harvested and processed for LUC activity.

24). The loss of IL-1 or LPS inducibility precludes our ability to use this assay system to link pellino-2 to the control of either IL-1- or LPS-induced Rel-dependent gene expression. However, it is notable that in contrast to the wild-type or AP-1-mutated IL-8 promoter constructs, IL-1- or LPS-induced activity of the NF- κ B-mutated IL-8 promoter was equivalent to or slightly greater in the presence of the pellino-2 antisense construct. Taken together, these data reveal that loss of IL-1- or LPS-dependent activation of the IL-8 promoter that occurs in the presence of the pellino-2 antisense construct is not dependent upon a functional AP-1 element; whether the NF- κ B and/or C/EBP β elements are required is less clear.

In summary, the data presented demonstrate that a mouse homolog of the *Drosophila* pellino protein is expressed during embryogenesis and in adult tissues that are involved in the innate immune response. Consistent with results obtained in *Drosophila*, an interaction between pellino-2 and the mouse homolog of pelle was detected. Further analysis revealed that IL-1 treatment stimulates the interaction between mPLK/IRAK-1 and pellino-2 and that a decline in the steady-state level of pellino-2 transcripts (and presumably protein) compromised IL-1-dependent activation of the NF- κ B-dependent IL-8 gene promoter. Taken together, these data extend those previously reported for the *Drosophila* pellino protein and suggest that pellino-2 may be a required component of the type I IL-1R- and TLR signaling pathways.

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