Characterization of Pellino2, a substrate of IRAK1 and IRAK4

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Abstract Interleukin-1 (IL-1) receptor-associated kinases (IRAKs) are central components of Toll/IL-1 receptor (TIR) signaling pathways. In an attempt to discover novel signal transducers in TIR signaling, we identified human Pellino2 as an interaction partner of IRAK4. Pellino2 interacts with kinase-active as well as kinase-inactive IRAK1 and IRAK4. Furthermore, Pellino2 is one of the first substrates identified for IRAK1 and IRAK4. Functional studies using overexpression or RNAi knock-down of Pellino2 suggest a role of Pellino2 as a scaffolding protein similar to Pellino1. However, unlike Pellino1, Pellino2 does not seem to activate a specific transcription factor, but links TIR signaling to basic cellular processes.

Key words: IL-1 signalling; IRAK; Pellino

1. Introduction

Toll/interleukin-1 (IL-1) receptor (TIR) family members are central components of host defense mechanisms in a variety of vertebrate and invertebrate species [1]. They play a crucial role in the switch from innate to adaptive immunity in mammals, either by the recognition of pathogens or as receptors for pro-inflammatory cytokines.

The TIR family can be divided into two subfamilies of transmembrane receptors. The members of one of these subfamilies, which include the IL-1 receptors, have immunoglobulin-like domains in the extracellular portion of the protein. The other subfamily is represented by the Toll-like receptors, whose extracellular domain is characterized by leucine-rich repeats [2]. The unifying structural motif of the TIR family is the so-called TIR domain in the intracellular domain [3,4].

The first step in TIR signaling is the ligand-induced formation of a receptor complex. In mammals, the close proximity of the TIR domains of the individual receptor chains allows the recruitment of the adapter molecules MyD88 [5,6] and Tollip [7]. MyD88 can then, in turn, recruit the IL-1 receptor-associated kinases IRAK1 and IRAK4 [8–11]. This complex interacts with the adapter TRAF6 [12]. The IRAK/TRAF6 interaction is a key step in the assembly of a multiprotein signalosome, which includes the mitogen-activated protein kinase (MAPK) kinase kinase TAK1 [13]. This complex activates a number of downstream signaling cascades, including IκB kinases, p38 and Jun N-terminal kinases (JNKs), leading to the activation of transcription factors such as NF-κB and AP-1 (for review see [1]).

Evolutionarily, the signal transduction mechanisms employed by TIRs are remarkably conserved [14]. In the fruit fly *Drosophila melanogaster*, Toll receptors recruit the adapter molecule Tube, and the Ser/Thr kinase Pelle, a homolog of human IRAKs [15]. Yeast two-hybrid screens with Pelle lead to the identification of *D. melanogaster* Pellino, a protein which can interact with wild-type, but not kinase-inactive Pelle, although it was not observed to be a substrate of Pelle [16]. In database searches, two human and murine homologs of Pellino were identified and cloned and were found to share about 60% identity and 75% similarity at the protein level [17,18]. Very recently, the first functional characterizations of human Pellino1 and murine Pellino2 were published [19,20]. Like their fruit fly counterparts, both proteins are able to interact with IRAKs, and both were implicated in IL-1-induced NF-κB activation.

Here we describe the characterization of human Pellino2. We identified Pellino2 in a yeast two-hybrid screen with human IRAK4. h-Pellino2 was found to be effectively phosphorylated by both IRAK1 and IRAK4, and in contrast to results published for m-Pellino2, h-Pellino2 does not seem to be an IL-1-specific activator of NF-κB. In contrast, h-Pellino2 could be a scaffolding molecule linking TIR signaling to general cellular processes involved in gene expression.

2. Materials and methods

2.1. Biological reagents and cell culture

The anti-Flag monoclonal antibodies M2, biotinylated M2 (M2-bio) and M2 cross-linked to Sepharose beads (M2 beads) as well as Flag peptide were purchased from Sigma-Aldrich. Rabbit anti-*myc* polyclonal antibody was purchased from Santa Cruz Biotechnology. KB cells, 293 cells and the 293 subline 293RI (stably overexpressing IL-1RI [11]) were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. His-tagged recombinant IRAK4 and IRAK4 KK213AA were expressed in insect cells by using a Gibco Bac-to-Bac baculovirus expression system, and recombinant Flag-tagged IRAK1 and IRAK1 K239S were expressed in insect cells by using the Baculogold system from PharMingen (following the manufacturer's recommendations). Synthetic siRNA for Pellino2 (AF302502: AAGGCTATCAGCTGCAAAGGTdTT) and control siRNA (TTCTCCGAACGTGTCACGTdTT) were purchased from Xeragon (Qiagen).

2.2. Cloning and expression vectors

Human IRAK4, IRAK4 KK213AA (kinase-inactive mutant) and IRAK1, IRAK1 K239S (kinase-inactive mutant) have been described elsewhere [9]. Human Pellino2 cDNA was obtained by polymerase chain reaction (PCR) with a universal cDNA library as template.

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Abbreviations: EMSA, electrophoretic mobility shift assay; IL-1, interleukin-1; IRAK, IL-1 receptor-associated kinase; TIR, Toll/IL-1 receptor

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For mammalian expression, N-terminal Flag-tagged Pellino2 was constructed by inserting the PCR-generated cDNA fragment in the mammalian expression vector pCMV-Tag2 (Stratagene).

2.3. Yeast two-hybrid screen

Yeast two-hybrid screens were performed using the Matchmaker GAL4 Two Hybrid System 3 (yeast host strain AH109; Clontech) and the human HeLa Matchmaker cDNA Library (Clontech) following the manufacturer's recommendations. Human IRAK4 was cloned into the pGBKT7 vector. Plasmid DNA from positive yeast clones was prepared by chloroform extraction and afterwards amplified in *Escherichia coli* for subsequent DNA sequencing.

2.4. Immunoprecipitations of overexpressed proteins and immunoblotting

For co-precipitation of overexpressed proteins, 5×10^6 293 cells were plated on 10 cm dishes and transfected on the following day with Lipofectamine2000 reagent (Invitrogen). Lysis (250 mM NaCl, 10 mM HEPES pH 7.9, 20 mM β-glycerophosphate, 1 mM sodium orthovanadate, 1 mM EDTA, 5 mM *p*-nitrophenyl phosphate, 1 mM dithiothreitol, protease inhibitors (Complete, Roche Molecular Biochemicals), 0.1% NP-40 and 10% glycerol) and immunoprecipitation were performed as described elsewhere [21].

2.5. In vitro kinase assays

N-terminal Flag-tagged Pellino2 was overexpressed in 293 cells and immunoprecipitated with M2 beads. Pellino2 was eluted from the beads with Flag peptide and incubated with 100 ng of recombinant kinases in 20 μl kinase buffer (20 mM Tris pH 7.6, 1 mM dithiothreitol, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 20 mM *p*-nitrophenyl phosphate, 1 mM EDTA, 1 mM sodium orthovanadate, protease inhibitors (Complete, Roche Molecular Biochemicals), 20 μM ATP) with 10 μCi γ[^{32}P]ATP (NEN) for 20 min at 30°C. The kinase reaction was stopped by boiling in 20 μl 2×SDS sample buffer. Proteins were separated via SDS–PAGE, and gels were dried and exposed to X-ray film.

2.6. Reporter assays

1×10^5 cells were seeded into 24 well plates. For experiments with the NF-κB-dependent ELAM promoter and the Jun/Fos-dependent AP-1 promoter, cells were transfected on the following day using Lipofectamine2000 (Invitrogen) with 0.01 μg p-37tkRL (*Renilla* luciferase gene under the control of the truncated HSV-TK promoter from position −37 to +52 [22]) and 0.1 μg pELAM-luc (3×NFκB binding sites [23]) or pAP-1-luc (4×AP-1 binding sites in front of a truncated HSV-TK promoter from −50 to +10) and 0.24 μg of expression constructs and 0.45 μg siRNA to a total of 0.8 μg DNA/RNA per transfection. After 48 h, the cells were harvested and *Renilla* and firefly luciferase activity was determined with the Dual-Luciferase Assay System (Promega). The PathDetect *trans*-reporting systems (Stratagene) were used according to the manufacturer's protocol. Cells were harvested 24 h after transfection and *Renilla* and firefly luciferase activity was determined with the Dual-Luciferase Assay System (Promega).

3. Results

3.1. Interaction of Pellino2 with IRAKs

In an attempt to identify novel proteins involved in TIR signaling, we performed yeast two-hybrid screens with full length IRAK4 as bait and a HeLa cDNA library as target. Three yeast clones specifically interacted with IRAK4 under high stringency conditions. Two of these three clones encoded proteins known to interact with IRAK4 (MyD88 and IRAK1), while the third one encoded the N-terminus (the first 167 of 420 amino acids) of Pellino2.

We isolated full length Pellino2 from a universal cDNA library via PCR, and verified the interaction with IRAK1 and IRAK4 in mammalian cells. For this purpose, we co-expressed Flag-tagged Pellino2 and *myc*-tagged IRAKs in 293 cells, immunoprecipitated Pellino2 and analyzed the immunoprecipitates for co-precipitating IRAKs (Fig. 1). Pellino2 is able to interact with both IRAK1 and IRAK4 independently of their kinase activities (IRAK1 K239S and IRAK4 KK213AA have point mutations in their ATP binding pockets, which abolish their kinase activities, Fig. 1A). At least under overexpression conditions in 293 cells, the interaction between Pellino2 and IRAK1 is significantly stronger than the interaction with IRAK4 (the left panel of Fig. 1A,C is from a longer exposure, the right panel from a shorter exposure of one Western blot). Western blots from cell lysates, which co-express Pellino2 with IRAK1, reveal two bands in the Pellino2 Western blot (Fig. 1D), suggesting an IRAK1-induced modification of Pellino2 resulting in a second Pellino2 species with

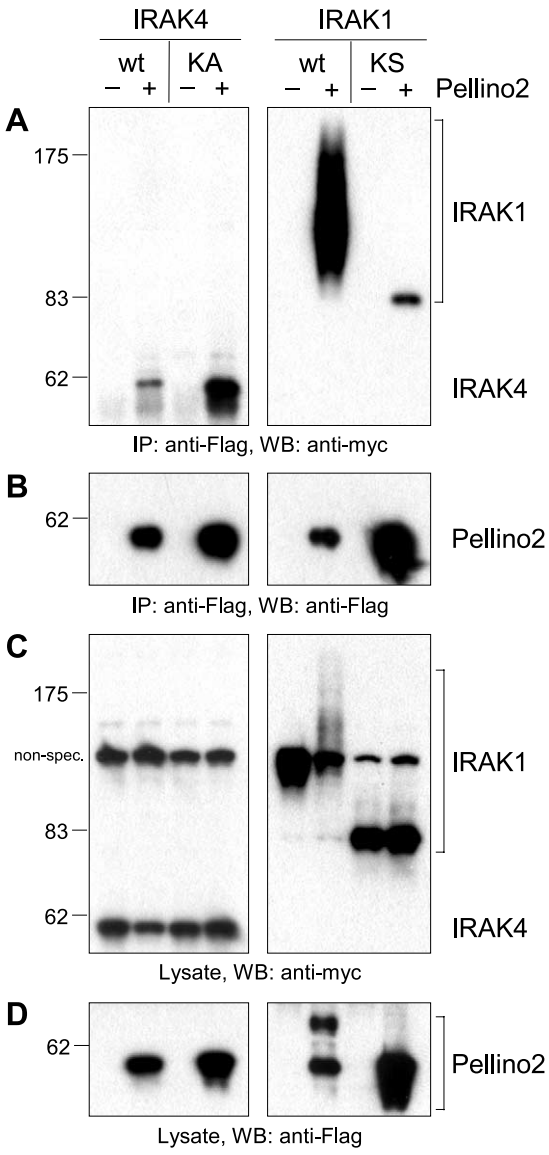

Fig. 1. IRAK1 and IRAK4 co-immunoprecipitate with Pellino2 in 293 cells. 293 cells were co-transfected with N-terminal *myc*-tagged IRAK4 (wt, kinase-active), IRAK4 KK213AA (KA, kinase-inactive mutant), IRAK1 (wt, kinase-active), IRAK1 K239S (KS, kinase-inactive mutant) and empty vector or N-terminal terminal Flag-tagged Pellino2. After anti-Flag immunoprecipitation of Pellino2, co-precipitating IRAKs were identified by anti-*myc* Western blot (A). Anti-Flag Western blot showed immunoprecipitated Pellino2 (B). Western blots of whole cell lysates with anti-*myc* antibody (C) and anti-Flag antibody (D) verified IRAK and Pellino2 expression.

a reduced electrophoretic mobility. This modification depends on the kinase activity of IRAK1 and cannot be achieved by co-expression of IRAK4 and Pellino2. This second Pellino2 species cannot be immunoprecipitated via the N-terminal Flag tag (Fig. 1B). The exact nature of the modification(s) leading to this dramatic shift in electrophoretic mobility is unknown. Both Pellino2 and IRAK1 have decreased expression levels when co-expressed, compared to expression alone or expression with IRAK4, and the number of high molecular weight species for IRAK1 is increasing, suggesting increased ubiquitinylation and subsequent degradation of IRAK1 [24] (Fig. 1C,D).

3.2. Pellino2 phosphorylation by IRAKs

We performed in vitro kinase assays with recombinant Pellino2 purified from 293 cells and IRAKs purified from insect cells. The Pellino2 preparation as well as preparations of kinase-inactive IRAK4 and IRAK1 did not contain any detectable kinase activity, while kinase-active IRAK4 and IRAK1 were clearly detectable by their autophosphorylation (Fig. 2A, left side). When Pellino2 was mixed with IRAKs, both IRAK4 and IRAK1 were able to efficiently phosphorylate Pellino2 (Fig. 2A, right side). Phosphorylation of Pellino2 by IRAK1 produced two distinct phosphoproteins, presumably hypo- and hyperphosphorylated Pellino2. This pattern is similar to that observed in co-expression experiments in 293 cells (Fig. 1D), although under the in vitro conditions only a small fraction of the Pellino2 could have been converted into

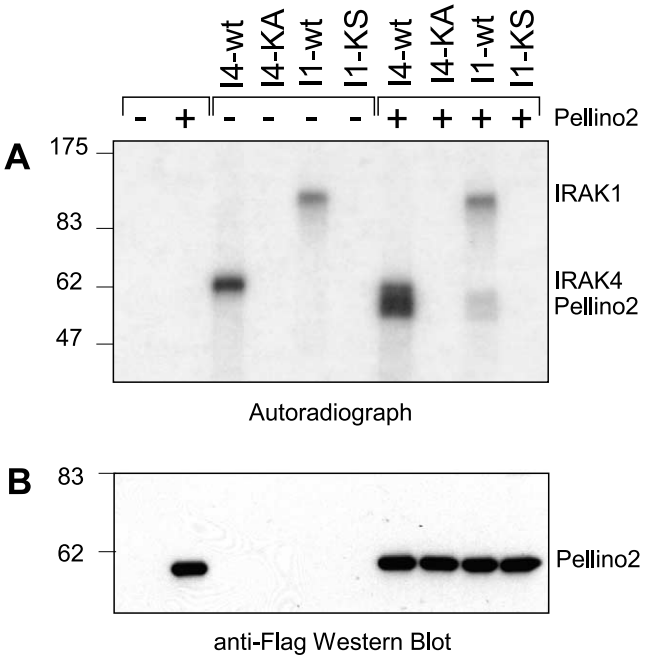

Fig. 2. Recombinant, kinase-active IRAK1 and IRAK4 phosphorylate Pellino2 in vitro. The in vitro kinase reaction was performed with 100 ng recombinant IRAK4 (I4-wt, kinase-active), IRAK4 KK213AA (I4-KA, kinase-inactive mutant), IRAK1 (I1-wt, kinase-active) or IRAK1 K239S (I1-KS, kinase-inactive mutant) expressed in insect cells and Pellino2 expressed in mammalian cells in the presence of 20 μM ATP and 10 μCi $\gamma[^{32}P]$ATP per reaction (A) or cold ATP only (B). The autoradiograph in A shows the autophosphorylation of the kinase-active IRAKs as well as the phosphorylation of Pellino2, whereas the anti-Flag Western blot of the non-radioactive in vitro kinase reaction shows equal amounts of Pellino2 per sample (B).

the hyperphosphorylated form, because it was not detectable by Western blot (Fig. 2B). In addition, the extent of the reduction in electrophoretic mobility of Pellino2 observed in this in vitro phosphorylation assay is much smaller than that observed in the co-immunoprecipitation experiment (Fig. 2), suggesting that the Pellino2 modification induced by co-expression with IRAK1 is not caused exclusively by phosphorylation. Despite the fact that under co-expression conditions the interaction between Pellino2 and IRAK4 seems to be weaker than the Pellino2/IRAK1 interaction (Fig. 1A), the Pellino2 phosphorylation by IRAK4 is at least as strong as the phosphorylation by IRAK1.

3.3. Functional characterization of Pellino2

Based on the observation that Pellino2 interacts with and is phosphorylated by IRAKs, we started to study the effects of Pellino2 overexpression or downregulation by RNAi [25] in the context of IL-1 signaling. When we overexpressed Pellino2 in 293RI cells, we were unable to detect any reproducible effects (positive or negative) on IL-1-induced NF-κB activation (reporter assays and electrophoretic mobility shift assay (EMSA)), JNK or Erk activation (in vitro kinase assays, anti-phospho Western blotting, *trans*-reporter assays) or AP-1 activation (EMSA, reporter assays). Similarly, downregulation of endogenous Pellino2 by about 80% (determined by Taqman qPCR, data not shown) in a RNAi approach did not have any significant effects on IL-1-induced cytokine production in 293RI and KB cells (IL-6 and IL-8, both enzyme-linked immunosorbent assay). Furthermore, both basal and IL-1-induced NF-κB activation (EMSA, reporter assay) were not affected in 293RI cells (data not shown).

The only effect we observed consistently was a Pellino2 overexpression-induced, increased luciferase reporter activity in 293RI cells (Fig. 3). Qualitatively, this increase in luciferase reporter activity was independent of the luciferase gene (Fig. 3A: *Renilla*, Fig. 3B: firefly luciferase) and independent of specific transcription factor binding sites in the promoter. In fact, the effects observed with a basic TATA box promoter from the HSV-TK promoter (Fig. 3A) were very similar to effects observed with AP-1/TK-driven promoters (Fig. 3B). Additionally, Pellino2 overexpression-induced activation of an AP-1/TK reporter construct could also be observed in murine embryonic fibroblast lines, although we could not detect any increased AP-1 DNA binding activity by EMSA (data not shown).

While reporter constructs with a basic promoter from HSV-TK responded strongly to Pellino2 overexpression (Fig. 3A,B), basic promoters derived from the adenoviral E1B gene responded moderately (Fig. 3D, here fused with a Gal4 binding site), whereas a promoter derived from the human ELAM gene did not respond significantly (Fig. 3E).

The Pellino2-induced, increased reporter activity could be completely reversed by Pellino2-specific siRNAs, suggesting that these effects are not general overexpression artifacts, but specific for Pellino2 activity (Fig. 3A–C).

4. Discussion

Here we report the characterization of human Pellino2, which we identified via its ability to interact with human IRAK4 in a yeast two-hybrid system. The first Pellino protein was identified in *D. melanogaster* as an interaction partner of

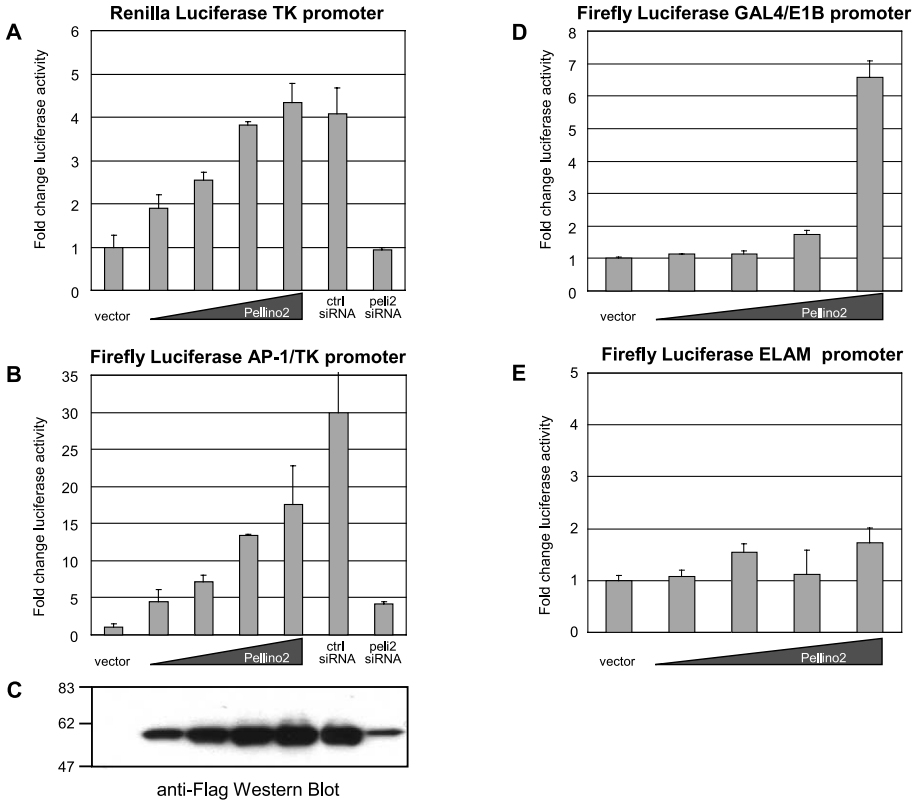

Fig. 3. Pellino2 overexpression enhances luciferase reporter activity. A–C: 293RI cells were transfected in triplicate with vectors encoding *Renilla* luciferase under the control of the truncated HSV-TK promoter and firefly luciferase under the control of 4×AP-1/TK promoter together with increasing amounts of Flag-tagged Pellino2 as well as unspecific (ctrl siRNA) or Pellino2-specific siRNA (peli2 siRNA). Cells were harvested 48 h after transfection and the activities measured for *Renilla* luciferase (A) and firefly luciferase (B). Expression of Pellino2 and its reduction due to the Pellino2-specific siRNA was verified by anti-Flag Western blotting of cell lysates (C). D: Cells were transfected in triplicate with firefly luciferase under the control of GAL4/E1B promoter and pFA2-CREB fusion *trans*-activator plasmid and increasing amounts of Flag-tagged Pellino2. Cells were harvested 24 h after transfection and the *Renilla* and firefly activities were measured. E: Cells were transfected and harvested as in B, but with a firefly luciferase construct under the control of a truncated ELAM promoter.

the Ser/Thr kinase Pelle [16]. Two homologs were described in man and mouse [17]. Similar to *D. melanogaster* Pellino, human Pellino1 and murine Pellino2, the human Pellino2 is able to interact physically with Ser/Thr kinases of the Pelle/IRAK family [19,20]. In contrast to dm-Pellino, h-Pellino2 can interact with IRAK1 and IRAK4 independently of their kinase activity, and most importantly, h-Pellino2 is one of the first kinase substrates identified for IRAK1 and IRAK4.

For Pellino1, a model was proposed in which Pellino1 serves as a scaffolding protein in an intermediate complex at the plasma membrane, allowing IRAKs and TRAF6 to leave the IL-1 receptor (complex I) and to interact with the TAK1/TAB1/TAB2 complex (complex II) [19]. It can be speculated that Pellino2 serves a similar function. This speculation is supported by the observation that co-expression of Pellino2 and kinase-active IRAK1 results in a high molecular weight species of Pellino2, which cannot be immunoprecipitated under standard conditions, indicating either a dramatic conformational change or change in the subcellular location of Pellino2, e.g. into membrane rafts. The phosphorylation of Pellino2 by activated IRAK1 and IRAK4 could trigger and modulate the translocation of IRAKs from complex I to complex II.

Both Pellino2 and IRAK1 show a decreased expression level when co-expressed in 293 cells. The IRAK1 Western blot indicates an increase in high molecular weight species (Fig. 1C), suggesting increased ubiquitinylation and degradation of IRAK1 [24]). However, we did not observe any effects of the Pellino2/IRAK interaction on the kinase activity of IRAKs in vitro when these assays were performed with recombinant proteins, which suggests that Pellino2 is not a direct modulator of IRAK1's or IRAK4's kinase activity, at least under these experimental conditions.

The experimental evidence linking Pellino proteins to TIR signaling in *Drosophila*, mice and man is very strong. Their ability to interact with Pelle/IRAKs, and the lack of any domain capable of enzymatic activity, makes a function as scaffold or adapter protein very likely. Nevertheless, the biological effects of this scaffolding or adapter function are less clear. Human Pellino1 was shown to be specifically involved in IL-1-induced NF-κB activation in human 293 cells: increased levels of Pellino1 (overexpression) lead to an increase in basal NF-κB activation, while a decrease in Pellino1 expression (RNAi) leads to a decrease in IL-1-induced NF-κB activation [19]. On the other hand, murine Pellino2 overexpression was shown not to have any effect on basal, IL-1- or lipopolysaccharide (LPS)-induced reporter gene activation, although reduction of m-Pellino2 expression levels in an antisense approach leads to a decrease in IL-1- and LPS-induced IL-8 reporter gene activity in a murine embryonic fibroblast cell line [20].

Theoretically the different observations obtained in published studies of h-Pellino1 or m-Pellino2 as well as our observations regarding h-Pellino2 could be explained by species- and cell type-specific functions of Pellino molecules. Especially comparing Pellino1 and Pellino2, completely redundant functions are unlikely, despite their 81% identity and 90% homology. The observed differences between human and murine Pellino2 are more difficult to explain, and might be more a result of different experimental procedures than of different biological functions.

In our hands, the suppression of endogenous Pellino2 levels by 80% in human 293 and KB cells does not have any significant effects on IL-1-induced IL-8 production or reporter gene activation. In addition, we cannot observe any reproducible effects of h-Pellino2 overexpression on MAPK activation. But Pellino2 overexpression was found to consistently and reproducibly increase the luciferase activity in most reporter assays, independently of the specific luciferase gene or specific transcription factor binding sites, and without increasing the DNA binding activity of the transcriptions factors AP-1 and NF-κB. The activation of luciferase activity depended more on the basic promoter used for a particular luciferase construct than on the regulatory elements of this promoter, although the extent of the activation was, of course, not completely independent of specific transcription factor binding sites. This hints towards a more general role of Pellino2. The high degree of evolutionary conservation of Pellino molecules from different species (h-Pellino2 is 60% identical and 75% similar to dm-Pellino) also makes a highly specialized function unlikely [17,18]. Based on the observed lack of any specific effect of h-Pellino2 on tumor necrosis factor- or IL-1-mediated signaling pathways, Pellino2 is most likely not responsible for the IL-1-induced activation of a certain transcription factor, but links TIR signaling to general cellular functions, like the transcription machinery.

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