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Pellino3, a Novel Member of the Pellino Protein Family, Promotes Activation of c-Jun and Elk-1 and May Act as a Scaffolding Protein

Liselotte E. Jensen* and Alexander S. Whitehead

Toll-like receptors and the IL-1R are part of the innate immune response aimed at mobilizing defense mechanisms in response to infections or injury. These receptors can initiate common intracellular signaling cascades. One intermediate component in these signaling cascades is Pellino, which was first identified in Drosophila and shown to interact with IL-1R-associated kinase. Two homologues, Pellino1 and Pellino2, have been identified in mammals. A novel member of the Pellino protein family has been identified and named Pellino3. Pellino3 shares 84 and 85% amino acid identity with Pellino1 and Pellino2, respectively. Two alternatively spliced Pellino3 mRNAs, Pellino3a and Pellino3b, are widely expressed. Pellino3 physically interacts with IL-1R-associated kinase-1, TNF receptor-associated factor-6, TGFB-activated kinase-1, and NF-kB-inducing kinase in an IL-1-dependent manner, suggesting that it plays a role as a scaffolding protein. In reporter assays Pellino3 leads to activation of c-Jun and Elk-1, but not NF-kB. Pellino3 also leads to activation of c-Jun N-terminal kinase. These data suggest that Pellino3 plays an important role in the innate immune response. The Journal of Immunology, 2003, 171: 1500–1506.
Additional adapter proteins and kinases have been proposed to be components of the TLR/IL-1 signaling cascade (reviewed in Ref. 2). One of these is NF-κB-inducing kinase (NIK), which was originally considered to be involved in the activation of NF-κB (15). Later studies using NIK knockout mice and mice carrying the alymphoplasia mutation in the NIK gene suggested that NIK is expendable in the signaling pathways activated by IL-1 (16), but do not exclude its involvement. NIK has also been linked to the processing of p100, the precursor of p52, which is one of the subunits of NF-κB (17). In addition, NIK can induce phosphorylation of MEK1, ERK1, and ERK2 (18, 19).

Pellino was first identified in Drosophila as a protein that binds to Pelle, the Drosophila homologue of IRAK (20). Due to its binding affinity for IRAK, Pellino has, since its discovery, been considered to play a role in TLR/IL-1 signaling (20); however, its precise function has remained largely undefined. Two homologues of Pellino have been identified in mammals, Pellino1 and Pellino2 (21, 22). Two recent studies have shown that human Pellino1 (23) and mouse Pellino2 (24) are required for activation of NF-κB. Both studies showed, as expected from previous work with Drosophila (20), that the mammalian Pellino1 and Pellino2 proteins interact with IRAK1 (23, 24). The human Pellino1 has further been shown to interact with IRAK4 and TRAF6 (23). Here we describe the identification of a novel Pellino protein, which we have named Pellino3, and establish that it interacts with IRAK1, TRAF6, TAK1, and NIK. We further show that Pellino3 can activate the MAPK pathway.

Materials and Methods

Computer analyzes

Computer searches of EST databases were performed through the National Center for Biotechnology Information (NCBI, Bethesda, MD) search launcher. The Conserved Domain Database and Conserved Domain Architecture Database (NCBI); the Simple Modular Architecture Research Tool (EMBL, Heidelberg, Germany); and Motif Scan in a Protein Sequence program (Swiss Institute of Bioinformatics, Basel, Switzerland) were used to search for conserved protein domains. Protein alignments were performed using the Multiple Alignment Program (Human Genome Sequencing Center, Baylor College of Medicine, Houston, TX) and Boxshade 3.21 (Swiss Institute of Bioinformatics, ISREC, Switzerland). Predictions of secondary structure were performed via the Computer analyzes program (Swiss Institute of Bioinformatics, Basel, Switzerland). Predictions of secondary structure were performed via the Computer analyzes program (Swiss Institute of Bioinformatics, Basel, Switzerland).

3’RACE and cloning of Pellino3

Primers were designed based on GenBank entries BE263635, BE266502, and BE799573. Two sequence-specific forward primers, 5’-AGCAC CATCTCCCGCTATGC-3’ and 5’-GCAGCTCATTGCCGCTACACG-3’, were used for 3’RACE with the 5’3’RACE System (Roche, Indianapolis, IN) according to the manufacturer’s instructions. The Pellino3 cDNAs were amplified using the GC-RICH PCR System (Roche) according to the manufacturer’s instructions. The Pellino3 coding sequences were cloned into the mammalian expression vectors pcDNA4/HisMax (Invitrogen, Carlsbad, CA) and p3XFLAG-CMV-14 (Sigma-Aldrich, St. Louis, MO). Dual-tag expression constructs were generated by first cloning the Pellino3 coding sequence into pcDNA4/HisMax and subsequently subcloning the His-tag-Pellino3 coding sequence into p3XFLAG-CMV-14.

Northern blotting and RT-PCR

A Northern blot of human poly(A) RNA isolated from different tissues (OriGene Technologies, Rockville, MD) was hybridized overnight with an α-32PdCTP-labeled probe generated by PCR amplification of 127 bp of the 5’ N-terminal encoding region from the Pellino3 cDNA (positions 13–139 in GenBank entry AF487546 and 13–139 in GenBank entry AF487546 in ULTRAhyb solution (Ambion, Austin, TX) at 42°C, washed three times in 0.1× SSC/0.1% SDS at 50°C, and subjected to autoradiography. The blot was subsequently re-probed with an α-32PdCTP-labeled β-actin cDNA probe (OriGene Technologies) and visualized as described above.

Human tissue total RNA was obtained from Clontech (Palo Alto, CA). Reverse transcription of 1 μg of total RNA was performed at 42°C using AMV reverse transcriptase (Promega, Madison, WI), oligo(dT), primer (Amersham Pharmacia Biotech, Piscataway, NJ), and Anti-RNase RNase inhibitor (Ambion). Fragments derived from the Pellino3a and Pellino3b cDNAs (646 and 574 bp, respectively; positions 13–658 in GenBank entry AF487546 and positions 13–586 in GenBank entry AF487547) were amplified in 40 cycles of PCR using a common set of primers and AmpliTaq Gold (Roche) according to the manufacturer’s instructions. PCR products were analyzed on SYBR Green I (Molecular Probes, Eugene, OR)-stained 8% polyacrylamide (37.5/1, acrylamide/bis-acrylamide) gels using STORM and ImageQuant technology (Molecular Dynamics, Sunnyvale, CA).

Cloning and expression vectors

The MyD88, IRAK1, NIK, and TRAF6 constructs were described previously (9). Portions of Pellino3a and Pellino3b coding sequences, obtained by PCR amplification, were cloned into pcDNA4/HisMax. The coding sequence of TAK1 (alternative splice variant A, GenBank accession no. AB009356) was cloned in pcDNA4/HisMax. The integrity of all constructs was confirmed by sequencing.

Cell lines and cultures

HepG2 human hepatoma cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in DMEM (MediaTech, Herndon, VA) supplemented with 10% FCS (Gemini Bio-Products, Woodland, CA), 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 50 μg/ml gentamicin (Life Technologies, Grand Island, NY). Human 293 embryonic kidney cells were obtained from American Type Culture Collection and maintained in DMEM supplemented with 10% FCS and 50 μg/ml gentamicin. Cells were treated with 10 ng/ml of IL-1α (National Cancer Institute, Frederick, MD). All experiments were performed at least three times.

Immunoprecipitation and Western blotting

Total proteins were extracted as described previously (9). Flag-tagged proteins were immunoprecipitated in Anti-FLAG HS, M2 ab-coated, 96-well plates (Sigma-Aldrich) at 4°C for 4 h. Wells were rinsed three times (5–20 s) and were washed three times (5–10 min) in Dulbecco’s PBS with magnesium and calcium, and 1% Nonidet P-40. Proteins bound to the wells were eluted in 65% lysis buffer, 100 mM DTT, 2 M urea, 0.85% SDS, and 2.5% glycerol for 10 min. Proteins were separated in NuPAGE 4–12% Bis-Tris gels (SDS-PAGE; Invitrogen) and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech).

Endogenous proteins were immunoprecipitated, with target-specific Abs as indicated, at 4°C for 2 h. Complexes and free Abs were captured by adding 15 μl (dry volume) of protein A-agarose beads (Invitrogen) and incubating for an additional 2 h. Agarose beads were washed five times (5–10 min) in Dulbecco’s PBS with magnesium and calcium, and 0.1% Nonidet P-40. Bound proteins were eluted in 65% lysis buffer, 100 mM DTT, 2 M urea, 0.85% SDS, and 2.5% glycerol. Proteins were separated in NuPAGE 4–12% Bis-Tris gels (SDS-PAGE; Invitrogen) and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech). Membranes were blocked in PBS/5% nonfat milk. Proteins were detected using specific primary Abs. Primary Abs were visualized using HRP-conjugated goat anti-rabbit Ig or goat anti-mouse Ig and ECL Western blotting detection reagents (Amersham Pharmacia Biotech).

Kinase assays

Total protein was extracted from cells, and JNK was immunoprecipitated as described above using anti-JNK2 (FL; Santa Cruz Biotechnology, Santa Cruz, CA). Washed immunoprecipitates were resuspended in kinase buffer (20 mM Tris (pH 7.6), 1 mM DTT, 20 mM MgCl2, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na3VO4, 50 mM NaF, 20 mM para-nitrophenylphosphate, 10% SDS, and 50 μM ATP) and separated into two aliquots. One aliquot was used for quantitation of JNK protein immunoprecipitated using Western blotting. The second aliquot was used for the actual JNK-kinase assay. GST-ATF2 (1 μg; gift from Dr. F. Coluccio Leskow) and 10 μCi of [γ-32P]ATP (Amersham Pharmacia Biotech) were added, and samples were incubated at 37°C for 30 min. Reactions were stopped by addition of 100 mM DTT, 2 M urea, 0.85% SDS, and 2.5% glycerol and boiling for 5 min. Proteins were separated by SDS-PAGE and visualized using autoradiography.

Reporter assays

A minimal E-selectin promoter (positions 1409–1579 in GenBank accession no. AF540378) containing a single NF-κB site was cloned into pGL2-Basic (Promega). The Renilla luciferase reporter construct (pRL-null) was obtained from Promega. The PathDetect c-Jun and Elk-1 Trans-Reporting Systems were obtained from Stratagene (La Jolla, CA) and used according

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to the manufacturer’s instructions. In brief, 50 ng of the luciferase reporter plasmid pFR-Luc, in which the expression of luciferase is driven by a GAL4-inducible promoter, was cotransfected into 2.5 × 10^5 cells together with 5 ng of pFA2-c-Jun, encoding the trans-activating fusion protein GAL4 (DNA binding domain residues 1–147)-c-Jun (residues 1–223), 5 ng of pRL-null, and variable amounts of Pellino3 expression constructs. Control cells were transfected with pFC2-dbd (encoding only the GAL4 DNA binding domain residues 1–147) instead of pFA2-c-Jun. If a protein under investigation either directly or indirectly phosphorylates c-Jun, this leads to dimerization of the GAL4-c-Jun fusion protein, which then binds to the GAL4 promoter in pFR-Luc and induces the expression of luciferase. For Elk-1 assays cells were transfected with pFA2-Elk-1, encoding the trans-activating fusion protein GAL4 (DNA binding domain residues 1–147)-Elk-1 (residues 307–428), instead of pFA2-c-Jun. In experiments in which cells were transfected with varying amounts of expression vectors, the absolute amount of DNA used in each individual transfection was held constant by cotransfecting appropriate amounts of empty vector. For luciferase reporter assays cell lysates were assayed for luciferase and Renilla luciferase activity according to the manufacturer’s instructions (Dual-Luciferase Reporter Assay System; Promega). Reporter assays were performed in duplicate or triplicate.

Results and Discussion

Identification of two splice variants of a novel Pellino protein

A search of the NCBI EST database to identify potential novel Pellino proteins with homology to Pellino1 and Pellino2 isolated entries BE263635, BE266502, and BE799573. Resch et al. (footnote in Ref. 22) have independently observed homology between these GenBank entries and human Pellino1 and Pellino2. Alignment of the three ESTs revealed partial overlaps (not shown), indicating that they were most likely derived from the same transcript. The composite sequence obtained from the alignment contained a presumptive 5′-untranslated region, start codon, and open, but incomplete (i.e., the stop codon is missing), reading frame. Based on the composite sequence, forward primers for use in 3′ RACE were designed. An ~800-bp PCR product was isolated and sequenced to reveal a 143-bp overlap with the established sequence tag (EST)-derived composite sequence together with 654 bp that were not present in BE263635, BE266502, or BE799573. This additional sequence encodes the C-terminal end of a novel gene product, which we have named Pellino3, and also contains the 3′-untranslated region, including the poly(A) signal and poly(A) tail.

Based on sequence derived from the EST clones and the above PCR product, primers were designed to permit amplification of the full-length coding region from reverse-transcribed mRNA and cloning into mammalian expression vectors. Several clones were sequenced to confirm identity with that of the EST/3′RACE chimera. Interestingly, several clones had an identical 72-bp deletion, indicating the likelihood that the Pellino3 gene transcript is alternatively spliced. The larger splice variant was designated Pellino3a, and the smaller splice variant was designated Pellino3b; their sequences were submitted to GenBank under accession numbers AF487456 and AF4874573. Alignment of the protein sequences encoded by these putative alternative splice variants with those of the previously identified Drosophila and human Pellino proteins (Fig. 1) revealed a remarkable degree of conservation. Pellino3 is 84, 85, and 59% homologous to human Pellino1, human Pellino2, and Drosophila Pellino, respectively. The alignment also revealed that both Pellino3 splice variants are alternatively spliced. The larger splice variant was found in Pellino3a, but not in Pellino3b (Fig. 1), confirming that Pellino3a and Pellino3b represent two differentially spliced products of a single gene. The positions of Pellino3 exons correspond to those of PEL11 and PEL12 (Fig. 1), suggesting that all three have evolved from a common ancestral gene.

Tissue distribution of Pellino3a and Pellino3b mRNA

The distribution of Pellino3 mRNA in human tissues was determined by Northern blot analysis using a probe directed against the 5′ region of Pellino3. A band of ~2.5 kb was detected at high levels in brain, heart, and testis and at lower levels in kidney, liver, lung, placenta, small intestine, spleen, and stomach (Fig. 2A, upper panel). Pellino3 mRNA was not detected in colon or muscle tissue (Fig. 2A, upper panel). The Northern blot was reprobed for β-actin mRNA to verify equal loading (Fig. 2A, lower panel). The upper band represents cytoplasmic actin, and the lower band represents the muscle-specific forms.

To determine whether low levels of Pellino3 mRNA are present in colon and muscle tissue and to examine the tissue distribution of the Pellino3a and Pellino3b alternative splice variants, RT-PCR analysis of human total RNA samples extracted from different tissues was performed. We determined in initial experiments, sampling aliquots during the exponential phase of amplification, that the ratio of the Pellino3a- and Pellino3b-derived cDNA products did not change during amplification; hence, band intensities within samples can be compared (not shown). The RT-PCR of the full panel of tissue RNA was performed into the plateau phase of amplification to ensure optimal sensitivity. Therefore, levels between samples cannot be compared. Pellino3 mRNA was also present in muscle and colon tissue using this RT-PCR approach, suggesting that the Pellino3 message is present in all tissues. With the exception of lung tissue, which only contained Pellino3b mRNA, both Pellino3 mRNA were present in all tissues examined, albeit at different relative levels (Fig. 2B).

Pellino3 promotes activation of c-Jun and Elk-1, but not NF-κB

IRAK1 and TRAF6 are well-characterized intermediates in the TLR/IL-1 signaling pathway leading to NF-κB activation (reviewed in Ref. 2). Overexpression of either factor leads to spontaneous activation of NF-κB. Therefore, the possibility that Pellino3 overexpression could induce the expression of an NF-κB–luciferase reporter was tested. Both HepG2 and 293 cells were transfected with the NF-κB–luciferase and Renilla luciferase reporters together with increasing amounts of Pellino3a or Pellino3b expression construct, and the resulting luciferase and Renilla luciferase activities were measured as described in Materials and Methods. Control cells were transfected with the reporter constructs together with constructs encoding either IRAK1 or TRAF6.
Both IRAK1 and TRAF6 induced NF-κB-driven luciferase expression, but neither Pellino3 protein had any effect on the level of luciferase expression (Fig. 3).

Elk-1 and c-Jun are activated by signaling subpathways involving MAPKs. To examine whether Pellino3 could initiate signaling through these pathways, cells were transfected with the PathDetect c-Jun and Elk-1 Trans-Reporting Systems, which have previously been used to evaluate the function of IRAK4 (6), together with increasing amounts of either Pellino3a or Pellino3b expression constructs, and the consequent luciferase and Renilla luciferase activities were determined. Both Pellino3 proteins promoted concentration-dependent increases in c-Jun and Elk-1 (Fig. 3) activities. In samples from cells transfected with the highest levels of Pellino3a and Pellino3b, 22- and 39-fold increases in c-Jun activity and 17- and 15-fold increases in Elk-1 activity were observed, respectively (Fig. 3).

Pellino3 promotes activation of JNK

Elk-1 and the AP-1 subunits, c-Jun and ATF2, are activated via phosphorylation mediated by JNK, which is itself activated by phosphorylation. To determine whether the Pellino3 proteins activated JNK kinase, cells were transfected with Pellino3a or Pellino3b expression constructs. Endogenous JNK was subsequently immunoprecipitated, and the ability to phosphorylate ATF2 was examined in vitro kinase assays. In both samples derived from cells transfected with Pellino3a or Pellino3b expression constructs, strong signals indicating phosphorylation of ATF2 were observed (Fig. 4). However, no signal was detected in the mock-transfected samples, demonstrating that Pellino3a and Pellino3b can both promote the activation of JNK activity.

Pellino3 interacts with IRAK1

Drosophila Pellino was originally identified as a protein binding to Pelle/IRAK (20). We therefore investigated whether Pellino3 could interact with IRAK1. Expression constructs encoding C-terminally Flag-tagged Pellino3 protein were cotransfected with IRAK1, IRAK1b (a naturally occurring alternative splice variant of IRAK1, which is kinase inactive) (9), or kiiIRAK1 (a kinase-inactive mutant of IRAK1). The Flag-tagged Pellino3 proteins were immunoprecipitated using anti-Flag, and coprecipitating proteins were identified using Western blotting after SDS-PAGE. Strong signals were observed in band positions corresponding to IRAK1a in immunoprecipitates from cells that had been cotransfected with the IRAK1a expression construct and either of the Pellino3 expression constructs (Fig. 5A). These signals were specific, since samples from cells that had been transfected with empty vector instead of Pellino3 expression constructs did not contain this band (Fig. 5A). In contrast, samples from cells transfected with the kiiIRAK1 mutant or IRAK1b expression constructs did not contain the respective IRAK1 proteins following immunoprecipitation (not shown). The latter is in agreement with similar observations previously made in the Drosophila system, in which it was
shown that Pellino only bound to the kinase domain of catalytically active forms of Pelle (20), the Drosophila homologue of mammalian IRAK.

**Pellino3 interacts with TRAF6, TAK1, and NIK**

MyD88 is an adapter protein involved in recruiting IRAK1 to the IL-1R or Toll receptor complexes. Three signaling intermediates downstream of IRAK1 are TRAF6, TAK1, and NIK (reviewed in Ref. 2). Since Pellino3 interacts with IRAK1a, experiments were performed to determine whether the two Pellino3 splice variants can interact with MyD88, TRAF6, TAK1, and NIK. Flag-tagged Pellino3a or Pellino3b was coexpressed with MyD88, TRAF6, TAK1, or NIK in 293 cells. The Flag-tagged Pellino3a or Pellino3b was immunoprecipitated, and proteins coprecipitating with either splice variant were identified by Western blotting analysis after SDS-PAGE. TRAF6 (Fig. 5B), TAK1 (Fig. 5C), and NIK (Fig. 5D), but not MyD88 (Fig. 5E), could be detected in communoprecipitated samples from cells cotransfected with either Pellino3 expression construct, but not in samples from control cells transfected with either a TRAF6, TAK1, or NIK expression construct and empty vector (Fig. 5). These data demonstrate that both Pellino3 proteins can directly interact with TRAF6, TAK1, and NIK.

**Pellino3 interacts with endogenous protein partners only after activation by IL-1**

Abs directed against and specific for mammalian Pellino proteins are currently not available, and the generation of such is hampered by both the high degree of evolutionary conservation and the existence of multiple, structurally related family members. Therefore, to determine whether Pellino3 can interact with the endogenous protein partners identified through the above screening, 293 cells were transfected with low levels (well below that which can trigger spontaneous activation of downstream signaling cascade, as described above, and 1/20th of that used in the experiments presented in Fig. 5) of an expression vector encoding Flag-tagged Pellino3a. Cells were treated with IL-1, and total lysates were harvested after 0, 15, 30, and 45 min. Lysates were divided into five aliquots, one of which was used to assess Pellino3a expression before immunoprecipitation, and others that were used to assess binding to each of the endogenous proteins (IRAK1, TRAF6, TAK1, or NIK). Endogenous proteins were immunoprecipitated, and any coprecipitating Pellino3a was identified using Western blotting and anti-Flag. In samples that had been immunoprecipitated with anti-IRAK1, anti-TRAF6, or anti-TAK1, Pellino3a could be detected 15 and 30 min after addition of IL-1, but not before addition of IL-1 (0 min) or after 45-min exposure to IL-1 (Fig. 6). These data suggest that there are transient IL-1-induced interactions with all

**Figure 2**

Expression of Pellino3a and Pellino3b mRNA in human tissue. A, Human tissue poly(A) RNA Northern blot (OriGene Technologies) was sequentially hybridized with [α-32P]dCTP cDNA probes directed against Pellino3 and β-actin as described in Materials and Methods. B, Human tissue total RNA was subjected to RT-PCR as described in Materials and Methods. The positions of the Pellino3a and Pellino3b PCR products are shown to the right.

**Figure 3**

Pellino3 promotes activation of c-Jun and Elk-1. Cells (2.5 × 10^5) were cotransfected with the pFC-Luc and Renilla luciferase reporter constructs plus the c-Jun activator/GAL4 fusion expression plasmid pFA2-c-Jun or the Elk-1 activator/GAL4 fusion expression plasmid pFA2-Elk-1 as described in Materials and Methods. For NF-κB-specific assays, cells were cotransfected with the minimal E-selectin-pGL2 and Renilla luciferase reporter constructs. Cells were additionally transfected with increasing amounts of Pellino3a (A) or Pellino3b (B) expression constructs. Total amounts of DNA transfected into cells were held constant by cotransfection with balancing amounts of empty vector. Luciferase values were standardized against Renilla luciferase to adjust for variations in transfection efficiencies. The standardized luciferase values are graphically represented as fold induction compared with values from cells transfected only with reporter constructs and empty vector (0 ng). Triplicate samples were analyzed, and SDs are shown with error bars.

**Figure 4**

Pellino3 promotes activation of JNK. Cells (1 × 10^5) were cotransfected with empty vector (mock) or Pellino3a or Pellino3b expression constructs. Cells were lysed, and the expression of Pellino3 proteins was verified using Western blotting with anti-Flag Abs (upper panel). Endogenous JNK was immunoprecipitated, and any coimmunoprecipitating Pellino3a was identified using Western blotting and anti-Flag. In samples that had been immunoprecipitated with anti-IRAK1, anti-TRAF6, or anti-TAK1, Pellino3a could be detected 15 and 30 min after addition of IL-1, but not before addition of IL-1 (0 min) or after 45-min exposure to IL-1 (Fig. 6). These data suggest that there are transient IL-1-induced interactions with all

**Figure 5**

Upper panel, cells were transfected with low levels of total DNA and empty vector (Mock). Cells were cotransfected with pFC-Luc and Renilla luciferase reporter constructs. Cells were additionally transfected with increasing amounts of Pellino3a or Pellino3b expression constructs. The standardized luciferase values are graphically represented as fold induction compared with values from cells transfected only with reporter constructs and empty vector (0 ng). Triplicate samples were analyzed, and SDs are shown with error bars. Lower panel, cells were transfected with low levels of total DNA and empty vector (Mock). Cells were cotransfected with the minimal E-selectin-pGL2 and Renilla luciferase reporter constructs. Cells were additionally transfected with increasing amounts of Pellino3a or Pellino3b expression constructs. The standardized luciferase values are graphically represented as fold induction compared with values from cells transfected only with reporter constructs and empty vector (0 ng). Triplicate samples were analyzed, and SDs are shown with error bars.
of the well-established intermediates of the TLR/IL-1 signaling cascade. Pellino3a was only detected after 45 min in the series of samples that had been immunoprecipitated with anti-NIK (Fig. 6), suggesting that Pellino3 engages in a sequential pattern of interactions involving NIK. Similar results were obtained using Flag-tagged Pellino3b and immunoprecipitation as described above (not shown). The observations that Pellino3 can interact directly with IRAK1, TRAF6, and TAK1 (Fig. 5) and that, in response to IL-1, such interactions appear to occur simultaneously (after 15–30 min; Fig. 6) suggest that Pellino3 may act as a scaffolding protein holding the functional signaling complexes together (see also below).

The results in Fig. 5, showing that protein interactions could be detected in the absence of IL-1 treatment, may at first appear to be in conflict with the results in Fig. 6, where such interactions were dependent upon the presence of IL-1. However, the formation of protein complexes is dynamic and reversible. According to Lecatelier’s principle, an increase in the concentration of one or both of the proteins involved would force the equilibrium toward complex formation. In the experiments depicted in Fig. 5, Pellino3 together with IRAK1, TRAF6, and TAK1 (Fig. 5) and that, in response to IL-1, such interactions appear to occur simultaneously (after 15–30 min; Fig. 6) suggest that Pellino3 may act as a scaffolding protein holding the functional signaling complexes together (see also below).

The results in Fig. 5, showing that protein interactions could be detected in the absence of IL-1 treatment, may at first appear to be in conflict with the results in Fig. 6, where such interactions were dependent upon the presence of IL-1. However, the formation of protein complexes is dynamic and reversible. According to Le-Chateliers principle, an increase in the concentration of one or both of the proteins involved would force the equilibrium toward complex formation. In the experiments depicted in Fig. 5, Pellino3 together with IRAK1, TRAF6, TAK1, or NIK were overexpressed, hence favoring complex formation in the absence of IL-1. Such forced formation of protein complexes may explain why it is possible to activate downstream signaling cascades via overexpression (as shown above for activation of c-Jun and Elk-1), i.e., functional signaling complexes are formed that can then activate downstream signaling components. In contrast, 20-fold less Pellino3 expression construct was used in the experiments depicted in Fig. 6 than in those shown in Fig. 5. Furthermore, only a single protein was expressed. Hence, conditions for the formation of complexes with endogenous proteins would remain unfavorable until activation of the signaling pathway by IL-1. Via mechanisms that are currently largely unknown, but may involve phosphorylation of signaling factors, conformational changes, and/or formation of scaffolds, the IL-1 signaling event leads to changes in the association and dissociation constants of the equilibrium, and protein complexes may consequently be formed even at low concentrations of the individual signaling factors.

Previously reported data regarding the branchpoint of the signaling subpathways that lead to activation of JNK and NF-κB have been contradictory. One study showed that different domains of IRAK1 are required for signaling through the different subpathways (12), suggesting that the pathways branch at or before
IRRK1. However, others have shown that dominant negative mutants of TAK1 prevent the activation of both NF-κB and JNK (25), suggesting a branchpoint at, or after, TAK1. Scaffold proteins appear to impose an architecture that physically separates signaling subpathway components, thereby providing the means for specific subpathway selection in the MAP signaling cascades (reviewed in Ref. 26). Given that neither Pellino3 protein can promote NF-κB activation, but that each can clearly promote the activation of c-Jun and Elk-1 and interact with IRAK1, TRAF6, and TAK1, it is likely that Pellino3a and Pellino3b act as scaffolding proteins that direct the above signaling components toward activation of c-Jun and Elk-1 instead of NF-κB. By introducing a third dimension to the TLR/IL-1 signaling cascade via scaffold proteins generating large multiprotein complexes, instead of adhering to the traditional view of the sequential engagement of components within a strictly linear cascade, it is possible to reconcile the previously reported conflicting results regarding branchpoints in the TLR/IL-1 signaling cascade. Thus, it is possible that IRAK1 can associate with several different scaffold proteins, including the Pellino proteins, and that different IRAK1 domains may be involved in such interactions. A fixed common step in signal transduction may be the association of IRAK1 with TRAF6, which leads to the engagement of TAK1. Thereafter, depending on which scaffolding protein(s) IRAK1 is already associated with, the mature signaling complex would be directed toward different subpathways with distinct end points, e.g., activation of NF-κB, c-Jun, or Elk-1. If the above general mechanism is essentially correct, the physical and chronological branchpoint would be at TRAF6/TAK1, but the commitment to a particular subpathway would have been established earlier in signaling complex formation at the time during which IRAK1 was itself activated. A previously reported study has demonstrated that activated IRAK1 is part of high molecular mass (600–800 kDa) multiprotein assemblies involving the IκB kinase complex (27), further supporting the above viewpoint.

Conclusion

Pellino proteins probably play an important role in establishing and maintaining the specificity of TLR/IL-1 signaling subpathways. Here we have described a novel Pellino protein, which we have named Pellino3, that can promote the activation of c-Jun and Elk-1. Furthermore, we have established that Pellino3 can interact with IRAK1, TRAF6, TAK1, and NIK in an IL1-dependent manner. The observation that Pellino3 apparently interacts with IRAK1, TRAF6, and TAK1 at the same time suggests a role for Pellino family members as scaffolding proteins that may control signaling branchpoint specificity. The differential expression of Pellino3 in different tissues may be a critical determinant of the cellular response to IL-1 or TLR ligands. Thus, tissues with low levels of Pellino3 may direct most of the incoming signal toward activation of NF-κB, whereas cells with higher levels may be able to support a relatively strong activation of the MAPK pathway.

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