Pellino2 activates the mitogen activated protein kinase pathway

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Abstract Engagement of the interleukin-1 (IL1) and Toll receptors, which are part of the innate immune system, facilitates activation of the transcription factors NF-κB, Elk-1 and AP-1. Pellino1 and Pellino2 have recently been shown to be intermediates in the pathway leading to activation of NF-κB. Here we demonstrate for the first time that human Pellino2 interacts with TRAF6 (tumor necrosis factor receptor associated factor 6) and that both Pellino1 and Pellino2 also interact with TAK1 (transforming growth factor-β activated kinase 1). We also show that Pellino2, but not Pellino1, can activate the mitogen activated protein kinase pathway leading to activation of AP-1 and Elk-1. These observations suggest a role for the Pellino proteins in the IL1/Toll signaling cascades as scaffold proteins that may regulate signaling branch-points.

Key words: Innate immunity; Cytokine; Interleukin-1; Signaling; c-Jun; Elk-1

1. Introduction

The innate immune system involves the local recognition of foreign agents such as bacterial lipopolysaccharide, peptidoglycan, DNA and viral double stranded RNA. These agents are specifically recognized by the Toll-like receptor family [1,2]. Engagement of the Toll-receptors triggers antimicrobial responses and the production of cytokines, including interleukin-1 (IL1). This in turn initiates the systemic acute phase response encompassing physiological changes that include fever, increased vascular permeability, hypotension, increased levels of circulating nitric oxide, infiltration of inflammatory cells and stimulation of the hypothalamic–pituitary–adrenal axis and corticosterone synthesis [3].

The Toll and IL1 receptors activate common intracellular signaling cascades [4]. A central consequence of signaling from the Toll and IL1 receptors is the activation of NF-κB, Elk-1, CHOP and AP-1 (homo- or hetero-dimers of cFos, cJun and/or ATF). These factors mediate the increased transcription of numerous genes and hence the increased synthesis of a range of proteins, many of which are involved in immunological responses. These include the kappa light chain of immunoglobulin, complement components, adhesion molecules and the major acute phase reactants, C-reactive protein and serum amyloid A protein [5].

The best described signaling cascade initiated by IL1 involves a trimeric protein complex of IL1, the IL1 receptor and IL1 receptor accessory protein [4]. This complex recruits the adapter protein MyD88 via the intracellular domains of the receptor and the accessory protein, an event that facilitates the subsequent recruitment of IL1 receptor associated kinase 1 (IRAK1) and IRAK4. The signaling cascade progresses through several downstream adapter proteins and kinases, including tumor necrosis factor receptor associated factor 6 (TRAF6), TRAF6 binding protein, two transforming growth factor-β activated kinase 1 (TAK1) binding proteins, and TAK1. TAK1 itself in turn activates the IκB kinase complex which contains the two IκB kinases IKK-α and IKK-β, as well as NF-κB bound to IκB [4]. IκB becomes phosphorylated and is degraded by the ubiquitin–proteasome pathway, after which NF-κB can translocate to the nucleus where it activates transcription by binding to specific sites in the promoters of numerous genes.

The signaling pathways which lead to the activation of AP-1, Elk-1 and CHOP are less well characterized. While some studies have suggested that these sub-pathways branch from the NF-κB activating pathway at, or after, TAK1 ([6] and references therein), others have suggested that they diverge at IRAK1 [7]. ECSIT (evolutionarily conserved signaling intermediate in Toll, [8]) pathways, mitogen activated protein (MAP) kinase/ERK kinase kinase 1 (MEKK1) and cJun N-terminal kinase (JNK) are sequentially engaged upstream activators of AP-1 [9], whereas Ras and p38 are involved in the activation of CHOP [10].

Many of the components of the IL1 signaling cascade are evolutionarily conserved across phyla. Investigations of the *Drosophila* signaling pathway have led to the identification of a novel protein, termed Pellino, that binds to Pelle (the homologue of IRAK) [11]. Two homologues of Pellino have been identified in mouse and humans [12,13]. It has recently been shown that mammalian Pellino1 and Pellino2 are required for activation of NF-κB [14,15], but the exact functions of these proteins remain poorly understood.

2. Materials and methods

2.1. Cloning and expression vectors

MyD88 and TRAF6 constructs are described elsewhere [16,17]. Pellino1 and Pellino2 cDNAs (GenBank accession nos. AF302505 and AF302502) were amplified by PCR and cloned into the mamma-

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Abbreviations: IL1, interleukin-1; IRAK, interleukin-1 receptor associated kinase; JNK, cJun N-terminal kinase; MAP, mitogen activated protein; TAK1, transforming growth factor-β activated kinase 1; TRAF6, tumor necrosis factor receptor associated factor 6

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lian expression vector p3XFLAG-CMV-14 (Sigma, St. Louis, MO, USA). The coding sequence of TAK1 (alternative splice variant A, GenBank accession no. AB009356) was cloned into pcDNA4/HisMax (Invitrogen, Carlsbad, CA, USA). The integrity of all constructs was confirmed by sequencing.

2.2. Cell line and cultures

Human embryonic kidney cells (293 cells) were obtained from the American Type Culture Collection and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and 50 μg ml^{-1} gentamicin (GibcoBRL, Grand Island, NY, USA). Cells were transfected as described elsewhere [17]. Cells were treated with 10 ng ml^{-1} IL1α (National Cancer Institute, Frederick, MD, USA) for 6 h. All experiments were performed at least three times with similar outcomes.

2.3. Western blotting and immunoprecipitation

Total proteins were extracted as described elsewhere [17]. Flag tagged proteins (100 μl) were immunoprecipitated in Anti-Flag® HS, M2 monoclonal antibody coated 96-well plates (Sigma) at 4 °C for 4 h. Wells were rinsed three times (5–20 s) and washed three times (5–10 min) in Dulbecco's PBS (phosphate-buffered saline) with magnesium and calcium, 0.1% NP-40. Proteins bound to the wells were eluted in 100 μl 65% lysis buffer, 100 mM dithiothreitol (DTT), 2 M urea, 0.85% sodium dodecyl sulfate (SDS), 2.5% glycerol for 10 min. Proteins were separated in NuPAGE 4–12% Bis–Tris gels (SDS–polyacrylamide gel electrophoresis (PAGE), Invitrogen) and transferred to Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Immunodetection was performed as described elsewhere [17].

2.4. Reporter assays

The renilla–luciferase reporter construct (pRL-null) was obtained from Promega. The PathDetect cJun, Elk-1 and CHOP *trans*-Reporting Systems were obtained from Stratagene (La Jolla, CA, USA) and used according to the manufacturer's instructions. In brief, 50 ng of the luciferase reporter plasmid pFR-Luc, in which expression of luciferase is driven by a GAL4 inducible promoter, was co-transfected into 2.5×10^5 cells with 5 ng pFA2-cJun (or pFA2-Elk1/pFA-CHOP) (encoding the *trans*-activating fusion protein GAL4-cJun (or GAL4-Elk1/GAL4-CHOP)), 5 ng pRL-null and variable amounts of Pellino expression constructs. Control cells were transfected with pFC2-dbd (encoding only the GAL4 DNA binding domain residues 1–147) instead of pFA2-cJun/pFA2-Elk1/pFA-CHOP. For experiments in which cells were transfected with varying amounts of expression vectors the total DNA used in each transfection was held constant by co-transfecting appropriate amounts of empty vector. For luciferase reporter assays luciferase and renilla–luciferase activities in cell lysates were measured according to the manufacturer's instructions (Dual-Luciferase Reporter Assay System, Promega). Reporter assays were performed in duplicate or triplicate.

2.5. Kinase assays

Total protein was extracted from cells and JNK immunoprecipitated as described above using anti-JNK2 (FL, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and protein A–agarose (Invitrogen). Washed immunoprecipitates were resuspended in kinase buffer (20 mM Tris, pH 7.6, 1 mM DTT, 20 mM $MgCl_2$, 20 mM β-glycerophosphate, 1 mM EDTA, 1 mM Na_3VO_4, 50 mM NaF, 20 mM *p*-nitrophenylphosphate, 10% protease inhibitor mixture and 5 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 JNK kinase assay. GST-ATF2 (1 μg, generous gift from Dr. Federico Coluccio Leskow) and 10 μCi of [γ-^{32}P]ATP (Amersham Pharmacia Biotech) were added and samples incubated at 37°C for 30 min. Reactions were stopped by addition of 100 mM DTT, 2 M urea, 0.85% SDS, 2.5% glycerol and boiling for 5 min. Proteins were separated by SDS–PAGE and visualized using autoradiography.

3. Results

3.1. Pellino1 and Pellino2 interact with TRAF6 and TAK1

Mammalian Pellino1 and Pellino2 have previously been shown to interact with IRAK1 [14,15]. Pellino1 has also been shown to interact with TRAF6 [14]. To determine whether Pellino1 and/or Pellino2 could interact with signaling factors in the IL1 pathway upstream and downstream of IRAK1, Flag tagged Pellino1 and Pellino2 were co-expressed with MyD88, TRAF6 or TAK1 in 293 cells. Flag tagged Pellino proteins were immunoprecipitated and proteins co-precipitating with the Pellino proteins analyzed using Western blotting. MyD88, which is upstream of IRAK1, was not detected after immunoprecipitation (not shown). In contrast, both TRAF6 and TAK1, which are downstream of IRAK1, were specifically co-precipitated with both Pellino1 and Pellino2 (Fig. 1) in samples derived from cells transfected with the respective Pellino expression construct, but not in samples derived from cells which had been transfected with empty vector. This establishes that there are specific direct interactions between the Pellino proteins and both TRAF6 and TAK1.

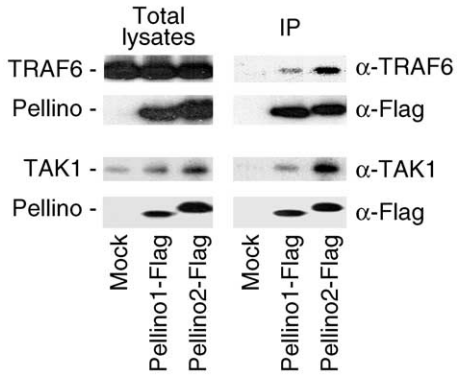

Fig. 1. Protein interactions with Pellino proteins. Cells were transfected with empty expression vector (*mock*) or expression vectors encoding Flag tagged Pellino1 or Flag tagged Pellino2. Cells were additionally transfected with an expression vector encoding TRAF6 (upper four panels) or TAK1 (lower four panels). Flag tagged proteins were immunoprecipitated, and total lysates and immunoprecipitates analyzed by Western blotting. Equal volumes of total lysates and eluted immunoprecipitated proteins were used. TRAF6, TAK1 and Pellino proteins were detected using anti-TRAF6 (H274, Santa Cruz Biotechnology), anti-TAK1 (M579, Santa Cruz Biotechnology) and anti-Flag (M2, Sigma), respectively.

3.2. Pellino2 promotes activation of cJun and Elk-1

The capacity of Pellino1 and Pellino2 to promote activation of the transcription factors cJun (one of the subunits of the AP-1 dimer), Elk-1 and CHOP, which are end-point components of IL1/Toll signaling sub-pathways distinct from that leading to NF-κB activation, was tested. Over-expression of increasing amounts of Pellino2 in the presence of the cJun and Elk-1 PathDetect reporter systems resulted in concentration dependent increases in luciferase activity (Fig. 2A). At the highest levels of transfected Pellino2 cDNA cJun and Elk-1 driven luciferase activity was increased eleven- and seven-fold, respectively. This strongly suggests that Pellino2 is involved in the signaling sub-pathways that lead to activation of AP-1 and Elk-1. In contrast, over-expression of Pellino1 had no such effect (Fig. 2B). Over-expression of Pellino2 did not affect the luciferase levels expressed using the CHOP PathDetect reporter system or the pFC2-dbd control plasmid encoding only the GAL4 DNA binding domain (Fig. 2A),

establishing that the effects of Pellino2 in the cJun and Elk-1 reporter systems are specific.

The above experiments were repeated in the presence of IL1. This cytokine treatment did not affect the degree of Pellino2 mediated cJun or Elk-1 activation, nor the inability of Pellino1 to mediate a similar effect (not shown).

We further tested if Pellino1 could inhibit or enhance the Pellino2 mediated cJun and Elk-1 activation. Cells were transfected with either the cJun or Elk-1 reporter systems, 135 ng Pellino2 expression construct and increasing amounts of Pellino1 expression construct. Co-expression of Pellino1 with Pellino2 did not affect the degree of Pellino2 mediated reporter activation under any of the test conditions (not shown).

3.3. Pellino2 activates JNK

Elk-1 and cJun are activated by phosphorylation. This event can be mediated by JNK, which itself is activated by phosphorylation. To examine whether Pellino1 and/or Pellino2 can activate JNK we performed in vitro kinase assays using ATP2, which is also activated by JNK, as a kinase substrate (Fig. 3). Only Pellino2 could activate the kinase activity of JNK, i.e. ATP2 was only phosphorylated in samples from cells over-expressing Pellino2. This observation is in agreement with our data using the PathDetect reporter system

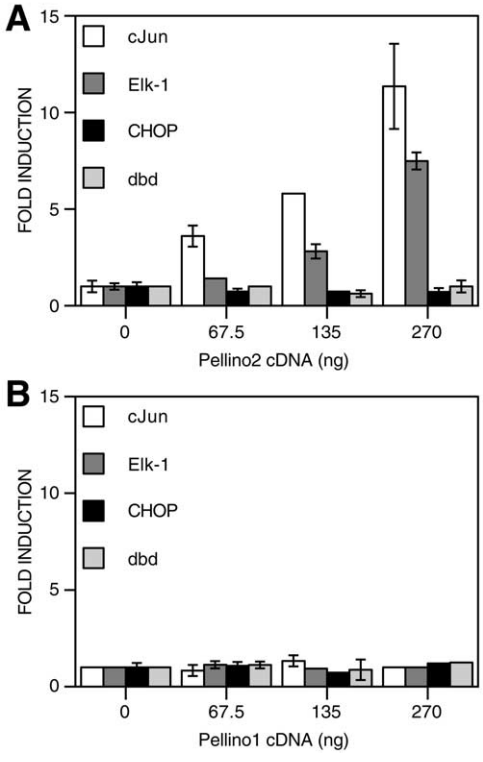

Fig. 2. Pellino2 activates cJun and Elk-1. Cells were co-transfected with pFC-Luc and renilla–luciferase reporter constructs plus the activator/GAL4 fusion expression plasmids pFA2-cJun, pFA2-Elk1 or pFA-CHOP or the control plasmid pFC2-dbd. Cells were additionally transfected with increasing amounts of Pellino2 (A) or Pellino1 (B) expression constructs. 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 to the values of cells transfected only with reporter constructs and empty vector (0 ng). Triplicate samples were analyzed and standard deviations are shown with error bars.

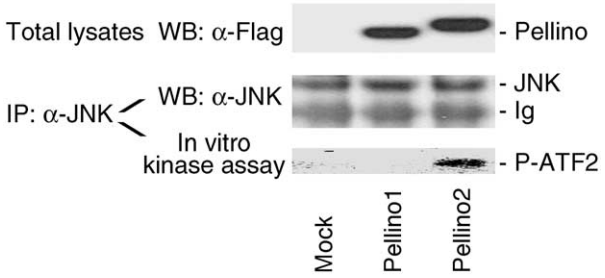

Fig. 3. Pellino2 can activate JNK. Cells were transfected with empty vector, Pellino1 or Pellino2 expression constructs. Cells were lysed and the expression of Pellino proteins verified using Western blotting with anti-Flag antibodies (upper panel). Washed JNK immunoprecipitates were separated into two aliquots, one of which was used to quantitate the amount of JNK immunoprecipitated (middle panel) and the other of which was used for in vitro kinase phosphorylation of the kinase substrate ATF2 (lower panel).

(Fig. 2), and establishes that Pellino2, but not Pellino1, activates the MAP kinase pathway.

4. Discussion

Pellino1 and Pellino2 have recently been shown to be involved in the Toll/IL1 signaling cascades leading to activation of NF-κB [14,15]. However the precise function of these proteins still remains to be determined. While both proteins have been shown to interact with IRAK1 [14,15], only Pellino1 has previously been shown to interact with TRAF6 [14]. Here we have demonstrated that Pellino2 also interacts with TRAF6. The dual interaction of Pellino1 and Pellino2 with IRAK1 and TRAF6 strongly suggests a role for the Pellino proteins in the IL1/Toll signaling cascade as either adapter or scaffold proteins. Given that it has been shown that IRAK1 can associate directly with TRAF6 [17,18], the latter may be a more accurate description. Several scaffolding proteins have been described for the MAP kinase signaling pathways (reviewed in [19]). The role of such scaffolding proteins is to assemble multiple signaling factors into functional protein complexes. Activated IRAK1 has previously been shown to be part of a large multiprotein complex (600–800 kDa) [20]. Our novel observation that both Pellino1 and Pellino2 also interact with TAK1 supports a role for the Pellino proteins as scaffolding proteins and suggests that they may hold IRAK1, TRAF6 and TAK1 together in an activated signaling-competent protein complex.

Protein interactions are often in a dynamic equilibrium. When a signaling event, such as binding of IL1 to its receptor, occurs the equilibrium may be shifted towards formation of protein complexes due to changes such as altered conformation or phosphorylation status. The ability of Pellino2 to facilitate 'spontaneous' activation of the MAP kinase pathway when over-expressed may be attributed to an induced assembly of functional signaling complexes of IRAK1, TRAF6 and TAK1. According to LeChâtelier's principle, the increased levels of Pellino2 would force the equilibrium towards complex formation even in the absence of an extracellular signaling event. Once functional signaling complexes are formed these could initiate downstream signaling events such as activation of JNK.

The branch-point at which the Toll/IL1 signaling sub-pathways leading to activation of NF-κB, AP-1 and Elk-1 diverge

is a matter of debate. Although one study has shown that different domains of IRAK1 are required for activation of the different sub-pathways, suggesting that the pathways diverge at IRAK1 [7], other studies, using dominant negative mutants of TAK1, have suggested that they diverge at, or after, TAK1 ([6] and references therein). These apparently contradictory results may be reconciled if, rather than considering the signaling pathway to be strictly linear, a third dimension involving scaffolding proteins is introduced. Upon activation IRAK1 interacts with TRAF6 and may also interact with several different scaffolding proteins, including Pellino1 and Pellino2. Different domains of IRAK1 may be involved in the interactions with different scaffolding proteins. TAK1 is then recruited into the protein complex. Depending on which scaffolding protein IRAK1/TRAF6/TAK1 is associated with, the protein complex will be directed towards different sub-pathways. Given the previously reported involvement of Pellino1 and Pellino2 in NF-κB activation [14,15] and our observations that Pellino2, but not Pellino1, can activate the MAP kinase pathway, Pellino1 may act as a scaffolding protein that directs and commits the signaling complex exclusively towards NF-κB activation, whereas Pellino2 may permit the complex to facilitate the engagement of both sub-pathways.

Here we have established that Pellino2, as well as Pellino1, interacts with TRAF6. We have also shown for the first time, that both Pellino proteins interact with TAK1. Furthermore, our results suggest that the Pellino proteins act as scaffolding proteins that differentially support the signaling sub-pathways leading to Elk-1, AP-1 and NF-κB activation, since Pellino2, but not Pellino1, can activate cJun and Elk-1. This underscores the potential importance of Pellino1 and Pellino2 as critical regulators of specificity in the IL1/Toll signaling cascades.

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