Pellino3 Is a Novel Upstream Regulator of p38 MAPK and Activates CREB in a p38-dependent Manner*†

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Received for publication, January 21, 2005, and in revised form, May 11, 2005
Published, JBC Papers in Press, May 25, 2005, DOI 10.1074/jbc.M500756200

This paper is available on line at http://www.jbc.org

Engagement of the interleukin-1 (IL-1) and Toll-like receptors triggers mitogen-activated protein kinase (MAPK) pathways and activation of transcription factors such as NFκB and AP-1. Recent studies have identified members of the Pellino protein family as novel mediators in mediating activation of these pathways. However, no evidence has been presented to date to suggest a role for the Pellino proteins in activation of the p38 MAPK pathway. We demonstrate herein that Pellino3 is a strong activator of p38 MAPK. RNA interference was used to reveal a physiological role for Pellino3 in the IL-1 pathway leading to activation of p38 MAPK. A series of N-terminal truncation and point mutants of Pellino3 were generated and tested for their ability to activate p38 MAPK in an effort to map sites of protein interaction important for p38 MAPK activation. In this way we show that the binding of Pellino3 to IL-1 receptor-associated kinase 1 coincides with its ability to promote p38 MAPK activation. TRAF-6 and transforming growth factor-β-activating kinase 1 are shown to act as downstream mediators of the activation of p38 MAPK by Pellino3. Finally, we confirm the functional consequences of the activation of p38 MAPK by Pellino3 by demonstrating that Pellino3 promotes translocation of the p38 substrate, MAPK-activated protein kinase2, from the nucleus to the cytoplasm and activates the transcription factor CREB in a p38 MAPK-dependent manner. Our study not only identifies Pellino3 as a novel upstream regulator of the p38 MAPK pathway but also probes the mechanistic basis underlying the ability of Pellino3 to promote activation of this pathway.

Human Toll-like receptors (TLRs) detect pathogen-associated molecular patterns (1–7) and share with the IL-1 receptor a conserved cytoplasmic Toll/IL-1R homology (TIR) domain (8). The engagement of these receptors allows for their TIR domains to recruit the adapter protein Myd88 (9) and other TIR domain-containing adapter proteins such as MyD88 adapter-like/TIR-domain-containing adapter protein (10, 11), TIR domain-containing adapter-inducing interferon-β/TIR-containing adaptor molecule-1 (12, 13), and TIR domain-containing adapter-inducing interferon-β-related adaptor molecule (14, 15). Some of these adapter proteins, such as Myd88, subsequently recruit and activate members of the IL-1 receptor-associated kinase (IRAK) family (16–20). The IRAK-Myd88 association triggers hyperphosphorylation of IRAK leading to its dissociation from Myd88 and its interaction with the downstream adaptor tumor necrosis factor receptor-associated factor 6 (TRAF-6) (21). This results in activation of transforming growth factor-β-activating kinase 1 (TAK-1) leading to stimulation of IκB kinases and ultimately activation of NFκB (22).

In addition to NFκB activation, IL-1R1 and TLRs can also initiate mitogen-activated protein kinase (MAPK) signaling cascades and activate multiple transcription factors. Thus IL-1 and LPS induce phosphorylation of p38, ERK1/2, and JNK (23–27). Although the mechanisms by which the MAPKs are activated by IL-1R1 and TLRs are incompletely understood, several upstream regulators have been identified, including those that mediate activation of NFκB. More specifically, TAK-1/TAB1 can activate the MAPK kinases MKK3/6 and MKK4, which in turn activate p38 and JNK, respectively (22).

Members of the novel Pellino protein family have recently emerged as mediators in transducing signals in the IL-1R/TLR pathways. Pellino was first identified in Drosophila by virtue of its association with Pelle, the Drosophila homologue of IRAK (28). Three mammalian homologues have since been identified (29–33). Mammalian Pellino1 interacts with IRAK, IRAK4, TRAF-6, and TAK-1 and is required for activation of NFκB (30) but is not involved in activation of the MAPK pathways (34). Mammalian Pellino2 also interacts with these same proteins (29, 35), and although there are contrasting reports on its involvement in activation of NFκB (29, 35), it has recently been shown to promote activation of ERK1/2 and JNK (34). Human Pellino3, both full-length and its alternative splice variant, interacts with IRAK, TRAF-6, and TAK-1, but interestingly fails to activate NFκB demonstrating that members of the Pellino family are not functionally redundant (31). However, like Pellino2, Pellino3 activates ERK1/2 and JNK. Although the latter MAPK pathways are activated by these Pellino proteins, no evidence has been presented to date implying a role for members of the Pellino family in activation of the p38 MAPK pathway. Indeed a role for Pellino1 and -2 in this pathway.
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FIG. 1. Pellino3L and Pellino3S activate the p38 MAPK pathway. A, HEK293 cells were co-transfected with the trans-activator plasmid pFA-CHOP, pRE-Luc (encoding GAL4-CHOP regulated firefly luciferase), phRL-TK (constitutively expressed Renilla luciferase) and designated amounts of plasmids encoding myc-tagged Pellino1, Pellino3S, or Pellino3L. Cell extracts were generated the following day and assayed for firefly (a measure of CHOP transactivation and hence p38 MAPK activity) and Renilla (for normalizing transfection efficiency) luciferase. Data are presented relative to cells transfected with empty vector (pTARGET) alone. Results represent mean ± S.E. of six independent experiments. Inset in A, HEK293 cells were transfected with plasmids encoding myc-tagged Pellino1, Pellino3S, or Pellino3L. Cell lysates were subjected to polyacrylamide gel electrophoresis and subsequently to Western immunoblotting using anti-myc antibodies. Immunoreactivity was visualized by enhanced chemiluminescence. B, HEK293 cells were transfected with various amounts of plasmids encoding Pellino3L (0.5, 1, and 1.6 μg), Pellino1 (1.6 μg), and MEK3 (1.6 μg). The pTARGET empty vector (EV) was used as a negative control. Cell lysates were generated the following day, subjected to polyacrylamide gel electrophoresis, and subsequently to Western immunoblotting using antibodies to detect levels of phosphorylated total p38 MAPK. C, HEK293 cells were co-transfected with a plasmid encoding wild-type p38 MAPK (1 μg) and Pellino3L, Pellino1, MEK3, or pTARGET empty vector (EV) (3 μg). Cell lysates were generated the following day and immunoprecipitated using phospho-specific p38 MAPK antibodies. Immunoprecipitates were incubated in kinase buffer with GST-ATF-2 and then subjected to Western immunoblotting using anti-phospho-ATF-2 monoclonal antibody.

way has already been excluded (31, 34). Because members of the Pellino family are not functionally redundant we explored the potential of Pellino3 to regulate the p38 MAPK pathway. We show that Pellino3 is a novel activator of this pathway and further demonstrate that Pellino3 activates CREB in a p38-dependent manner. By generating a series of truncation mutants of Pellino3 we map regions that positively influence p38 MAPK activity and identify a crucial amino acid residue in Pellino3 for IRAK1-Pellino3 association.

MATERIALS AND METHODS

Biological Reagents and Cell Culture—Anti-TRAF-6, anti-IRAK, and anti-TAK-1 were from Santa Cruz Biotechnology. Monoclonal anti-myc antibody was from Cell Signaling Technology. Constructs encoding TRAF-6, TRAF-6 DN (289–522), and IRAK-1 were from Tularik (San Francisco, Japan). Dominant negative TAK-1 (K66W) was a gift from Dr. H. Sakurai (Tanabe Seiyaku Co., Osaka, Japan). TRAF-6, TRAF-6 DN-(289–522), and IRAK-1 were from Tularik (San Francisco, CA). TAK-1 was a gift from Dr. Kunihiro Matsumoto (Nagoya University, Japan). The plasmids encoding wild-type and dominant negative p38 MAPK were gifts from Dr. Douglas T. Golenbock (University of Massachusetts Medical Center) and have been described previously (36).

Parental HEK293 cells and HEK293 cells stably expressing TLR4 (gift from Douglas T. Golenbock) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, penicillin G (100 μg/ml), and streptomycin (100 μg/ml). G418 (Geneticin, 0.5 mg/ml) was used as the selective agent for the stably transfected 293TLR4.

Cloning and Expression Vectors—Full-length and the alternative spliced variant of Pellino3 (designated Pellino3L and Pellino3S, respectively, in this report) were cloned from HEK293 cDNA. Pellino1 was cloned from human placental cDNA. Pellino2 was cloned from HEK293 cDNA. All cloning used the GC-RICH PCR system (Roche Applied Science) and primers for Pellino1, Pellino2, Pellino3L, and Pellino3S were based on sequences deposited in GenBankTM (accession numbers AF300987, NM_021255, AF487456, and AF487457, respectively). The genes were tagged at the N terminus with the c-myc epitope tag and subsequently cloned into the T/A cloning vector pTARGET (Promega Biological Reagents and Cell Culture—Anti-TRAF-6, anti-IRAK, and anti-TAK-1 were from Santa Cruz Biotechnology. Monoclonal anti-myc antibody was from Cell Signaling Technology. Constructs encoding TRAF-6, TRAF-6 DN (289–522), and IRAK-1 were from Tularik (San Francisco, Japan). Dominant negative TAK-1 (K66W) was a gift from Dr. H. Sakurai (Tanabe Seiyaku Co., Osaka, Japan). TRAF-6, TRAF-6 DN-(289–522), and IRAK-1 were from Tularik (San Francisco, CA). TAK-1 was a gift from Dr. Kunihiro Matsumoto (Nagoya University, Japan). The plasmids encoding wild-type and dominant negative p38 MAPK were gifts from Dr. Douglas T. Golenbock (University of Massachusetts Medical Center) and have been described previously (36).

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Liver and kidney, cDNA was subjected to RT-PCR using primers to selectively amplify regions of the Pellino3 and GAPDH cDNAs. This resulted in the generation of products 158- to 158-bp of lysate was retained for Western blot analysis of p38 MAPK phosphorylation—HEK293 cells (3 × 10^5/well) were seeded into 12-well plates and grown for 24 h. Cells were transfected, using Lipofectamine 2000, with a plasmid encoding wild-type p38 MAPK (1 µg) and Pellino3L, Pellino3Y44A (1.6 µg), and MEK3 (1.6 µg). The pTARGET empty vector was used as a negative control. Cells were harvested in SDS sample buffer the following day, resolved by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and probed for levels of phosphorylated and total p38 MAPK in the absence or presence of various concentrations of siRNA specific for Pellino3 or a scrambled control siRNA (5–15 nM). Cells were then transfected, using GeneJuice (Novagen), with MAPKAP kinase 2-DsRed (400 ng) in the presence or absence of Pellino3-pEGFP or Pellino3(Y44A)-pEGFP (400 ng). Cells were fixed in 4% paraformaldehyde for 15 min, washed three times with phosphate-buffered saline, and mounted with SlowFade anti-fade reagent (DAPI-containing medium, 1.5 µg/ml, Molecular Probes, Inc.). Confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets and analyzed using the LSM 5 browser imaging software.

RESULTS

Pellino3 Activates the p38 MAPK Pathway—To address the ability of Pellino3 to activate the p38 MAPK pathway, HEK293 cells were co-transfected with increasing amounts of expression plasmid encoding myc-tagged Pellino3L or Pellino3S and components of the PathDetect CHOP trans-Reporting System. The latter includes the GAL4-inducible promoter, pFR-Luc, and the trans-activating fusion protein GAL4-CHOP. Because CHOP is a substrate for p38 MAPK, and this leads to activation of its trans-activation capacity (37), the expression of luciferase from pFR-luc is an index of p38 MAPK activity. The overexpression of both Pellino3L and Pellino3S caused a dose-dependent induction of pFR-luciferase (Fig. 1A). Both forms exhibited comparable levels of activation with maximum stimulation of 6- to 7-fold. This represents a strong level of activation, because the maximum-fold induction affected by the positive MEK3 driver is 10- to 12-fold (data not shown). In contrast, the overexpression of Pellino1 failed to induce expression of luciferase (Fig. 1A) confirming its previously published inability to activate the p38 MAPK pathway (34). This differential ability of Pellino3 versus Pellino1 to activate the p38 MAPK pathway is not due to lack of expression of the latter, because Western immunoblotting of lysates confirmed expression of both constructs (Fig. 1A, inset).
Two additional independent assay systems were further employed to validate the CHOP reporter system and confirm that Pellino3 activates p38 MAPK. MEK3 was used as a positive driver in both assays. Firstly, cells were transfected with increasing amounts of an expression vector encoding Pellino3L, and cell extracts were assayed for levels of phosphorylated p38 MAPK using an antibody that detects doubly phosphorylated threonine 180 and tyrosine 182 of p38 MAPK. Cells showed some basal phosphorylation of p38 MAPK. However Pellino3L caused a dose-dependent increase in the levels of phosphorylated p38 MAPK, whereas Pellino1 was ineffective (Fig. 1B). As control for the assay, all samples showed equivalent levels of total p38 MAPK as judged by Western immunoblotting analysis using control antibodies that recognize p38 irrespective of its phosphorylation status.

An in vitro kinase assay for p38 MAPK was also used to further confirm its activation by Pellino3. Thus cells were transfected with Pellino3L, cell extracts were immunoprecipitated with phosphorylated p38 MAPK antibodies, and immunoprecipitates were assayed for their ability to catalyze the in vitro phosphorylation of the p38 MAPK substrate ATF-2. Immunoprecipitates from Pellino3L-transfected cells showed increased activity with respect to phosphorylation of ATF-2 (Fig. 1C). In contrast, Pellino1-transfected cells demonstrated comparable activity with cells transfected with empty vector. This assay, in conjunction with the other two systems, strongly confirms Pellino3 as a robust activator of p38 MAPK.

Deletion Analysis of Pellino3 Reveals Sites Important for p38 MAPK Activation—To address the structural basis underlying the unique ability of Pellino3, as the only member of the Pellino family, to activate the p38 MAPK pathway. The alignment of the protein sequences encoded by the mammalian Pellino genes demonstrates that greatest sequence divergence occurs at their N termini (Fig. 3A). Further, both forms of Pellino3 are longer at their N termini by 27 amino acids. Using Pellino3L as a reference, a series of truncation mutants were generated in an effort to identify key regions of importance to activating p38 MAPK. Emphasis was placed on regions that show greatest sequence divergence between Pellino3 and the other Pellino members. All mutants were assessed for their ability to activate p38 MAPK by IL-1, relative to a control Pellino3-scrambled siRNA (Fig. 2A). Neither siRNA had any effect on the levels of total p38 MAPK. To validate selective suppression of endogenous Pellino3 expression by the Pellino3-specific siRNA, RNA samples from siRNA-transfected cells were assayed for levels of Pellino3 and GAPDH mRNAs by reverse transcription-PCR analysis (Fig. 2B). The scrambled siRNA sequence had no effect on the mRNA levels of Pellino3 relative to cells that had been transfected in the absence of any siRNAs. In contrast the Pellino3-specific siRNA caused a strong suppression of Pellino3 mRNA expression and left barely detectable levels. Neither siRNA affected the levels of the housekeeping GAPDH mRNA.

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extracts were generated the following day and assayed for firefly (a measure of CHOP transactivation and hence p38 MAPK activity) and Renilla (for normalizing transfection efficiency) luciferase. Data are presented relative to cells transfected with empty vector (pTARGET) alone. Results represent mean ± S.E. of three independent experiments. B, HEK293 cells were transfected with plasmids encoding Pellino3L, Pellino3L Y44A, MEK3, or pTARGET empty vector (EV) (1.6 μg). Cell lysates were generated the following day and subjected to Western immunoblotting using antibodies to detect levels of phosphorylated and total p38 MAPK. C, HEK293 cells were co-transfected with a plasmid encoding wild-type p38 MAPK (1 μg) and Pellino3L, Pellino3L Y44A, MEK3, or pTARGET empty vector (EV) (3 μg). Cell lysates were generated the following day and immunoprecipitated using phospho-specific p38 MAPK antibodies. Immunoprecipitates were incubated in kinase buffer with GST-ATF-2 and then probed for the presence of TRAF-6, TAK-1, and IRAK. These studies demonstrated that full-length Pellino3 and the truncated/point mutant constructs described above all retained the ability to interact with IRAK, TRAF-6, and TAK-1 (31). Such interactions were lost for Pellino3-IRAK1 Interaction Coincides with Pellino3 Activation of p38 MAPK—The mechanistic basis to the capacity of Pellino3 and its mutated forms to activate the p38 MAPK pathway was next explored. Because it was previously shown that Pellino3 interacts with IRAK, TRAF-6, and TAK-1 (31), expression constructs encoding these same proteins were co-transfected with myc-tagged Pellino3 or truncated/mutated forms and examined for their potential to interact (Fig. 5). Pellino3 proteins were immunoprecipitated from cell lysates with anti-myc antibody, and the immunoprecipitates were probed for the presence of TRAF-6, TAK-1, and IRAK. These studies demonstrated that full-length Pellino3 and the truncated/point mutant constructs described above all retained the ability to interact with TRAF-6 (Fig. 5A) and TAK-1 (Fig. 5B). This implies that the binding site(s) on Pellino3 for these proteins lie downstream of amino acid 134. The interaction of the various Pellino3 proteins with IRAK was next assessed (Fig. 5C). Intriguingly full-length Pellino3 and the truncation mutants that retained the ability to activate p38 MAPK pathway co-precipitated with IRAK, whereas truncation mutants that were ineffective in the p38 MAPK assay failed to associate with IRAK. Furthermore, the site-directed mutagenesis of Y44A

![Fig. 4. Mutational analysis of tyrosine residue in the N terminus of Pellino3 abrogates its ability to activate p38 MAPK. A, HEK293 cells were co-transfected with the trans-activator plasmid pFA-CHOP, pFR-Luc (encoding GAL4-CHOP regulated firefly luciferase), phRL-TK (constitutively expressed Renilla luciferase), and designated amounts of plasmids encoding myc-tagged Pellino3L or site-directed mutants. Cell extracts were generated the following day and assayed for firefly (a measure of CHOP transactivation and hence p38 MAPK activity) and Renilla (for normalizing transfection efficiency) luciferase. Data are presented relative to cells transfected with empty vector (pTARGET) alone. Results represent mean ± S.E. of three independent experiments. B, HEK293 cells were transfected with plasmids encoding Pellino3L, Pellino3L Y44A, MEK3, or pTARGET empty vector (EV) (1.6 μg). Cell lysates were generated the following day and subjected to Western immunoblotting using antibodies to detect levels of phosphorylated and total p38 MAPK. C, HEK293 cells were co-transfected with a plasmid encoding wild-type p38 MAPK (1 μg) and Pellino3L, Pellino3L Y44A, MEK3, or pTARGET empty vector (EV) (3 μg). Cell lysates were generated the following day and immunoprecipitated using phospho-specific p38 MAPK antibodies. Immunoprecipitates were incubated in kinase buffer with GST-ATF-2 and then probed for the presence of TRAF-6, TAK-1, and IRAK. These studies demonstrated that full-length Pellino3 and the truncated/point mutant constructs described above all retained the ability to interact with TRAF-6 (Fig. 5A) and TAK-1 (Fig. 5B). This implies that the binding site(s) on Pellino3 for these proteins lie downstream of amino acid 134. The interaction of the various Pellino3 proteins with IRAK was next assessed (Fig. 5C). Intriguingly full-length Pellino3 and the truncation mutants that retained the ability to activate p38 MAPK pathway co-precipitated with IRAK, whereas truncation mutants that were ineffective in the p38 MAPK assay failed to associate with IRAK. Furthermore, the site-directed mutagenesis of Y44A...](Image 119x445 to 503x737)
abolished the ability of Pellino3 to interact with IRAK. The expression of IRAK and the various myc-tagged Pellino3 proteins was confirmed by Western immunoblotting of cell lysates (middle and bottom panels, respectively).

The above findings emphasize the crucial importance of Tyr-44 in Pellino3 for its ability to interact with IRAK. Interestingly this tyrosine residue is conserved in both Pellino1 and -2, and although both can interact with IRAK, neither can activate p38 MAPK. We explored the importance of the tyrosine residue in Pellino1 and -2 with respect to their interaction with IRAK (Fig. 6, A and B, respectively). Thus wild-type forms of both Pellino1 and -2 and their respective Y17A and Y19A mutants were assessed for their interaction with IRAK (Fig. 6). Interestingly the mutants mirrored their wild-type counterparts with respect to their ability to interact with IRAK suggesting that the tyrosine residue is of unique importance in Pellino3, whereas it is dispensable in Pellino1 and -2, at least with respect to facilitating interaction with IRAK.

**Activation of p38 MAPK by Pellino3 Is Mediated by TRAF-6 and TAK-1**—The downstream signaling components employed by Pellino3 in its activation of p38 MAPK was next addressed. As stated above, TRAF-6 and TAK-1 are known to act downstream of IRAK and upstream of p38, and these signaling molecules present themselves as lead candidates for mediating the activation of p38 by Pellino3. This was directly examined by measuring the regulatory effects of dominant negative forms of TRAF-6 and TAK-1 on Pellino3 activation of p38 MAPK in the GAL4-CHOP-based assay (Fig. 7). Both dominant negative constructs completely blocked the ability of Pellino3 to activate p38 MAPK indicating key roles for TRAF-6 and TAK-1 as downstream mediators of Pellino3 signaling.

**Pellino3 Triggers Translocation of MAPKAP Kinase 2 from the Nucleus to the Cytoplasm**—The functional consequence of p38 MAPK activation by Pellino3 was finally assessed. The best characterized substrate for activated p38 MAPK is MAPKAP kinase 2 (24). The latter is normally resident in the nucleus but translocates to the cytoplasm upon its phosphorylation and activation by p38 MAPK (38, 39). We thus investigated the potential of Pellino3 to promote the nuclear export of MAPKAP kinase 2. This was performed by the initial engineering of expression constructs encoding the fusion proteins MAPKAP kinase 2-DsRed (DsRed is a variant of Discosoma sp. red fluorescent protein) and Pellino3-pEGFP. HER293 cells were transfected with MAPKAP kinase 2-DsRed in the presence or absence of Pellino3-pEGFP, and cells were subsequently viewed by confocal microscopy. Cells, in which MAPKAP kinase 2-DsRed was expressed in the absence of Pellino3-pEGFP, showed red fluorescence with predominance in the nucleus (Fig. 8A, lower left panel) as evidenced by co-localization with the DAPI-stained nuclei (Fig. 8A, lower right panel). In contrast the co-expression of Pellino3-pEGFP showed a dramatic reduction of red fluorescence in the nucleus with concomitant increase in the cytoplasm of those cells that express Pellino3-pEGFP (Fig. 8B, lower left panel). Indeed the redistribution of MAPKAP kinase 2-DsRed from the nucleus to the cytoplasm manifested as yellow staining in the montage (Fig. 8B, lower right panel) due to the combined green and red fluorescence of Pellino3-pEGFP and MAPKAP kinase 2-DsRed, respectively.
Interestingly, Pellino3-pEGFP showed intense expression in as yet undefined structure(s) in the cytoplasm of the cell (Fig. 8B, upper right panel). We also designed a Pellino3(Y44A)-pEGFP fusion construct and co-transfected with MAPKAP kinase 2-DsRed. In contrast to wild-type Pellino3 the Y44A mutant failed to promote the nuclear export of MAPKAP kinase 2-DsRed (Fig. 8C, lower left panel). The lack of significant redistribution to the cytoplasm is evidenced by the absence of yellow fluorescence in the montage (Fig. 8C, lower right panel). This is consistent with the inability of Pellino3 (Y44A) to activate p38 MAPK. The expression of the Pellino3(Y44A)-pEGFP mutant was represented as a more diffuse pattern (Fig. 8C, upper right panel).
upper right panel) relative to Pellino3-pEGFP. Although the basis of this different pattern of expression is not understood at present, it is intriguing to speculate that the regions of intense expression of wild-type Pellino3 may represent the formation of macromolecular signaling complexes containing high concentrations of Pellino3.

**Pellino3 Activates CREB in a p38 MAPK-dependent Manner**—Finally the functional consequence of MAPKAP kinase 2 activation by Pellino3 was confirmed by examining the ability of Pellino3 to activate the transcription factor CREB. The latter is a substrate for MAPKAP kinase 2 (40). Both full-length and the alternative spliced variants of Pellino3 were overexpressed in HEK293 cells and measured for regulatory effects on expression of a co-transfected CREB-regulated reporter construct. Both forms induced expression of the luciferase reporter with maximal induction of 5- to 6-fold (Fig. 9A). In contrast Pellino1 was ineffective, and this is likely due to its inability to activate p38 MAPK. Indeed the role of p38 MAPK in mediating activation of CREB was confirmed by demonstrating that a dominant negative form of p38 inhibits the activation of the CREB reporter by Pellino3 (Fig. 9B). Furthermore, all of the Pellino3 mutants were examined for their regulation of CREB activity,
and this experiment demonstrated a perfect correlation between the ability to stimulate p38 MAPK and promote activation of CREB (Fig. 9C). This is reflected with the finding that the Y44A mutant is completely ineffective in activating CREB. These findings clearly show that activation of CREB is a downstream functional consequence of the stimulation of p38 MAPK by Pellino3.

DISCUSSION

This study demonstrates that Pellino3 is a novel upstream activator of the p38 MAPK pathway. By using RNA interference we also show a physiological role for Pellino3 in mediating IL-1 activation of p38 MAPK. In the Pellino protein family, the ability of Pellino3 to activate p38 MAPK appears to be unique, because a previous report has demonstrated that Pellino1 and -2 are ineffective in promoting activation of p38 MAPK (34). Indeed the present report confirms the inability of Pellino1 to activate this pathway. This study emphasizes that the members of the Pellino family are not functionally redundant, and the different members may play specific roles in activating selective pathways. It is interesting to note that both full-length and the alternative spliced variant of Pellino3 showed comparable efficacy in activating p38 MAPK and to date there are no known functional differences between the variants. The functional consequences of the activation of the p38 MAPK pathway by Pellino3 was also confirmed by demonstrating the nuclear export of MAPKAP kinase 2 in response to overexpression of Pellino3. MAPKAP kinase 2 is an immediate substrate for activated p38 MAPK, and the activation and nuclear export of MAPKAP kinase 2 are regulated by its phosphorylation (24, 38). The consequence of MAPKAP kinase 2 activation was also defined by demonstrating that Pellino3 activates the transcription factor CREB in a p38 MAPK-dependent manner. These studies thus identify a new signaling module consisting of upstream Pellino3 and the downstream sequential activation of p38 MAPK, MAPKAP kinase 2, and CREB.

Because Pellino3 is the only member of the Pellino family to promote activation of p38 MAPK, we attempted to identify unique characteristics of Pellino3 that may be associated with this activity. More specifically we compared the primary structure of Pellino3 with Pellino1 and -2, and the multiple sequence alignment clearly showed that most sequence divergence was present at their N termini. Furthermore, Pellino3 has an additional 27-amino acid region at its N terminus, and this was the lead candidate for conferring unique properties on Pellino3. However, the truncation of this region failed to affect the potential of Pellino3 to activate the p38 MAPK pathway, and thus the functional relevance of the region awaits further characterization. The next 15 amino acids in Pellino3 showed some sequence divergence with Pellino1 and -2. The truncation of this region showed a reduction in Pellino3 activation of p38 MAPK that would imply the existence of important residues within this region. However, the further truncation of the immediately adjacent KYGEL residues caused complete loss of p38 MAPK activation. The mechanistic basis to this finding was addressed by probing the potential of Pellino3 and its various mutants to interact with IRAK, TRAF-6, and TAK-1. These proteins were chosen as lead candidates, because they have previously been shown to interact with Pellino3 (31) and act as upstream regulators of p38 MAPK (22). The mutants that were ineffective in activating p38 were incapable of associating with IRAK, whereas full-length Pellino3 and its active mutants interacted with IRAK. The co-immunoprecipitation studies mapped the tyrosine residue Tyr-44 in Pellino3 as a critical residue for establishing protein-protein interactions with IRAK. It is highly unlikely that the inability of Y44A to interact with IRAK is due to nonspecific long range effects, because it retained the ability to interact with TRAF-6 and TAK-1. The co-immunoprecipitation assays indicate that the binding site(s) for TRAF-6 and TAK-1 are located at the C terminus or at least downstream of residue 134. Both TRAF-6 and TAK-1 are key downstream mediators of Pellino3, because their dominant negative counterparts abolished Pellino3 activation of p38 MAPK. Interestingly, Pellino1 and -2 have previously been shown to interact with IRAK, TRAF-6, and TAK-1 (30, 31, 35), but neither can activate p38 (34). Thus while the present studies propose that the interactions of Pellino3 with these signaling proteins are crucial for triggering activation of p38 MAPK, the ineffectiveness of Pellino1 and -2 would suggest that interaction with these proteins is not sufficient to drive the pathway. Pellino3 must interact with additional undefined proteins that contribute to its ability to engage the p38 pathway. Tyrosine residue 44 in Pellino3 is likely to be of major importance in facilitating such interactions. The present studies show that this tyrosine residue, although conserved in all members of the Pellino family, is uniquely important in Pellino3, at least with respect to facilitating interaction with IRAK. This tyrosine residue may also emerge to confer on Pellino3 the exclusive capacity to interact with other proteins that are necessary for triggering activation of the p38 MAPK pathway. Thus Pellino3 is likely to act as a scaffold protein that recruits a number of proteins to form a signaling complex, and the full complement of proteins in this complex is required to trigger downstream signaling.

Because tyrosine residues are subject to phosphorylation, we attempted to address the potential regulation of Pellino3 function by phosphorylation of Tyr-44. We thus mutated Tyr-44 to the phospho-mimetic residue glutamic acid. This mutant was inactive. Although this may immediately suggest that phosphorylation of Tyr-44 may lead to inactivation of Pellino3, the inactive nature of the Y44A mutant that cannot be phosphorylated at this residue would equivocate this proposal. Furthermore, although the side chain of glutamic acid mimics a phosphate group, it lacks the aromatic ring of tyrosine, and the phosphorylation of the ring may be crucially important.

In summary this study identifies Pellino3 as a novel upstream regulator of the p38 MAPK and emphasizes that members of the Pellino family are not functionally redundant. It also delineates the downstream effectors and consequences of Pellino3 signaling.

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