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

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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)[1] 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 adaptor 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 path-

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[1] The abbreviations used are: TLR, Toll-like receptor; TIR, Toll/IL-1R homology; IRAK, IL-1 receptor-associated kinase; TRAF-6, tumor necrosis factor receptor-associated factor 6; TAK-1, transforming growth factor-β-activating kinase; MAPK, mitogen-activated protein kinase; MAPKAP, MAPK-activated protein; DsRed, a variant of *Discosoma sp.* red fluorescent protein; IL-1, interleukin-1; IL-1R1, IL-1 type 1 receptor; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CHOP, CCAATT/enhancer-binding protein-homologous protein; GST, glutathione *S*-transferase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MEK, mitogen-activated protein kinase/extracellular signal-regulated kinase kinase; DN, dominant negative; IRES, internal ribosomal entry site; GFP, green fluorescent protein; EGFP, enhanced GFP; siRNA, small interference RNA; DAPI, 4′,6-diamidino-2-phenylindole.

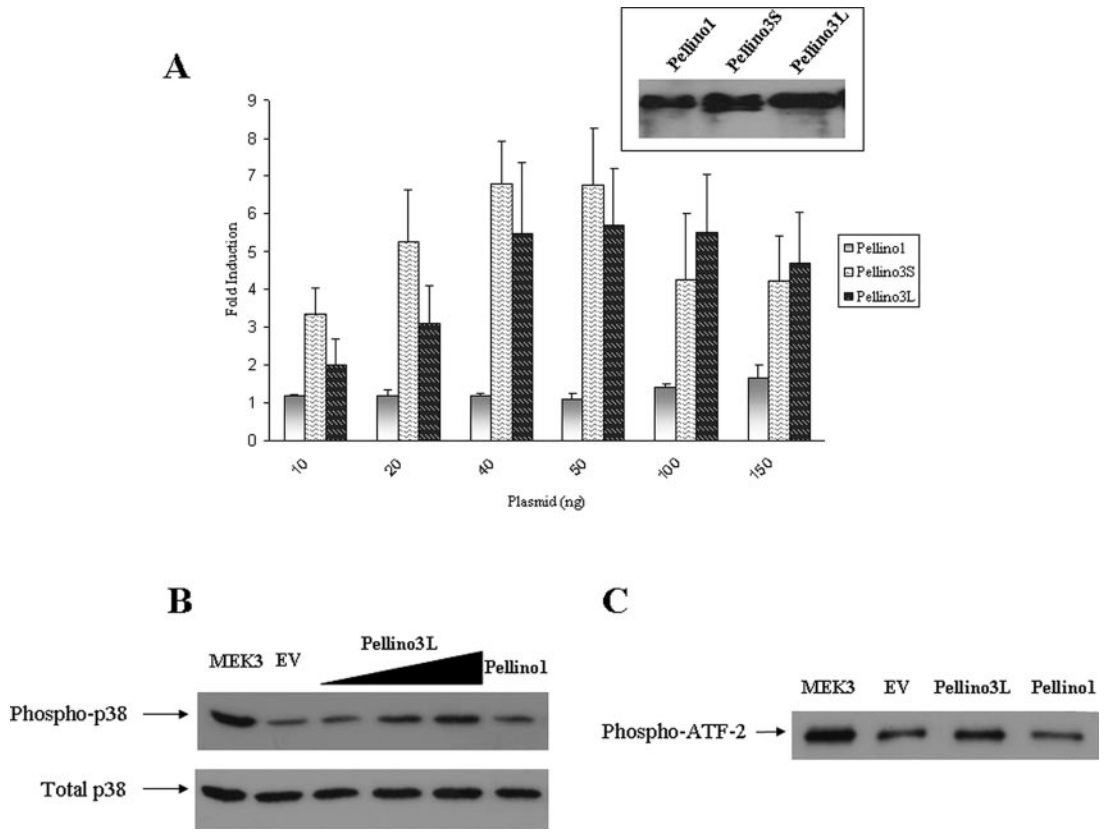


FIG. 1. **Pellino3L and Pellino3S activate the p38 MAPK pathway.** *A*, HEK293 cells were co-transfected with the *trans*-activator plasmid pFA-CHOP, pFR-Luc (encoding GAL4-CHOP regulated firefly luciferase), phRL-TK (constitutively expressed *Renilla* luciferase) and designated amounts of plasmids encoding *myc*-tagged 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 and 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, CA). TAK-1 was a gift from Dr. H. Sakurai (Tanabe Seiyaku Co., Osaka, Japan). Dominant negative TAK-1 (K66W) was a gift from Dr. Kunihiro Matsumoto (Nagoya University, Japan). The plasmids encoding wild-type and dominant negative p38 MAPK were gifts from Prof. R. J. Davis (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 GenBank™ (accession numbers AF300987, NM_021255, AF487456, and AF487457, respectively). The genes were tagged at the C terminus with the c-*myc* epitope tag and subsequently cloned into the T/A cloning vector pTARGET (Promega Biosciences, San Luis Obispo, CA) and a BamHI-XhoI-digested pcDNA3.1/Zeo (Invitrogen) mammalian expression vector. Truncation mutants of Pellino3L were generated by PCR using the GC-RICH PCR system and cloned into the pTARGET and pcDNA3.1/Zeo mammalian expression plasmids. Site-directed mutagenesis was performed using *Pfu* Turbo (Stratagene, La Jolla, CA), and mutants were generated according to the manufacturer's instructions. The integrity of all generated clones was confirmed by sequencing. The MAPK-activated protein (MAPKAP) kinase 2 (accession no. BC036060) was amplified by the GC-RICH PCR system, using HEK293 cDNA as template and cloned into a modified pIRES-DsRed expression plasmid (BD Biosciences, Clontech). The IRES was removed by a two-step process of first site-directed mutagenesis followed by the release of a restriction fragment containing the IRES thus allowing for the expression of a MAPKAP kinase 2-DsRed fusion protein. Pellino constructs were additionally subcloned into the EcoRI-digested pEGFP-N1 (BD Biosciences, Clontech) vector for confocal analysis.

CHOP Reporter System—The PathDetect CHOP *trans*-Reporting System (Stratagene) was used, according to the manufacturer's recommen-

dations, to measure activation of the p38 MAPK pathway. Briefly, HEK293 TLR4 cells (2.6×10^4 cells/well) were seeded into 96-well plates and grown for 24 h. Cells were then transfected, using GeneJuice transfection reagent (Novagen, Madison, WI), with firefly luciferase reporter plasmid pFR-Luc (60 ng), the *trans*-activator plasmid pFA-CHOP (activation domain of CHOP fused with the yeast Gal4 DNA binding domain, 1 ng), constitutively expressed *Renilla*-luciferase reporter construct (phRL-TK, 20 ng, Promega Biosciences), varying amounts of expression constructs. Total DNA was kept constant (230 ng/well) using the appropriate empty vector. The activation of CREB was assessed by performing equivalent transfections using CREB-regulated luciferase (60 ng, BD Biosciences, Clontech), phRL-TK plasmid (20 ng), and varying amounts of expression constructs. Cell extracts were generated 24 h later using the Reporter Lysis Buffer (Promega Biosciences), and extracts were assayed for firefly luciferase and *Renilla*-luciferase activity using the Luciferase Assay system (Promega Biosciences) and coelenterazine (1 μg/ml, Insight Biotechnology Ltd.), respectively.

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 (Invitrogen), with various amounts of plasmids encoding Pellino3L (0.5, 1, and 1.6 μg), Pellino1 (1.6 μg), Pellino3L Y44A (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 using the PhosphoPlus p38 MAP Kinase (Thr-180/Tyr-182) antibody kit as recommended by the manufacturer (New England Biolabs). Immunoreactivity was visualized by enhanced chemiluminescence.

Analysis of p38 MAPK Activity by in Vitro Kinase Assay—HEK293 cells (5×10^5/well) were seeded into 6-well plates and grown for 24 h. Cells were co-transfected, using Lipofectamine 2000, with a plasmid encoding wild-type p38 MAPK (1 μg) and Pellino3L, Pellino3L Y44A, Pellino1, MEK3, or pTARGET empty vector (3 μg). At 24-h post-transfection cells were lysed in 0.2 ml of lysis buffer (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.5% (v/v) Nonidet P-40, 50 mM NaF, 1 mM sodium orthovanadate, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, protease inhibitor mixture (25 μg/ml leupeptin, 25 μg/ml aprotinin, 1 mM benzamidine, 10 μg/ml trypsin inhibitor)). Lysates were then assayed for p38 MAPK activity using a non-radioactive p38α (SAPK2a kinase) activity assay kit as recommended by the manufacturer (Chemicon International). Briefly, cell lysates were 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.

Suppression of Endogenous Expression of Pellino3—Pre-designed small interfering RNA (siRNA) targeting Pellino3 was purchased from Ambion Inc. (target sequence, GAG GAC AGA CTG TTA ACA AAT), and a corresponding scrambled siRNA was designed and constructed using the *Silencer in Vitro* transcription siRNA construction kit (Ambion Inc.). HEK293TLR4 cells were seeded into 12-well plates (3×10^5 cells/well) and transfected 24 h later, using Lipofectamine 2000, with various concentrations of siRNAs (0–15 nM). Cells were treated 30 h following transfection with IL-1 (10 ng/ml) for 20 min and then harvested in SDS sample buffer. Samples were probed for levels of phosphorylated and total p38 MAPK as described above. To confirm suppression of endogenous expression of Pellino3, RNA was extracted from cells, using the RNAqueous-4PCR kit (Ambion Inc.) and subsequently reverse transcribed into cDNA using AMV-reverse transcriptase (Promega Biosciences). PCR was then performed on the cDNAs using GAPDH-specific primers (GAPDH-for, 5′-CCA TGC CAT CAC TGC CAC CCA GAA-3′; GAPDH-rev, 5′-GTC CAC CAC CCT GTT GCT GTA GCCG) and Pellino3-specific primers (Pellino3-for, 5′-GGC TTC GAT GCC TCT AGC AAC-3′; Pellino3-rev, 5′-CAG GCG GCA TGG AAA GCA TG-3′). PCR products were resolved on a 1% agarose gel.

Co-immunoprecipitation Analysis—HEK293 cells (5×10^5/well) were seeded into 6-well plates and grown for 24 h. Cells were transfected, using Polyfect (Qiagen), with the relevant Pellino construct (1.5 μg) in the presence or absence of expression plasmids encoding IRAK, TRAF-6, or TAK-1 (0.5 μg). At 24 h post-transfection cells were lysed in 0.5 ml of lysis buffer (as above). Cellular debris was removed by centrifugation at $12{,}000 \times g$ for 10 min. An aliquot (50 μl) of lysate was retained for Western blot analysis. Pre-cleared cell lysates were incubated overnight at 4 °C with monoclonal anti-*myc* antibody that had been pre-coupled with Protein A/G-Sepharose beads. Following incubation, the beads were centrifuged at $2{,}500 \times g$ for 5 min at 4 °C followed by five wash steps with 700 μl of lysis buffer lacking the protease inhibitor mixture. The beads were then resuspended in SDS sample buffer, separated by SDS-PAGE, transferred to polyvinylidene difluoride membranes, and analyzed by immunoblotting using the appropriate antibodies. Immunoreactivity was visualized by enhanced chemiluminescence.

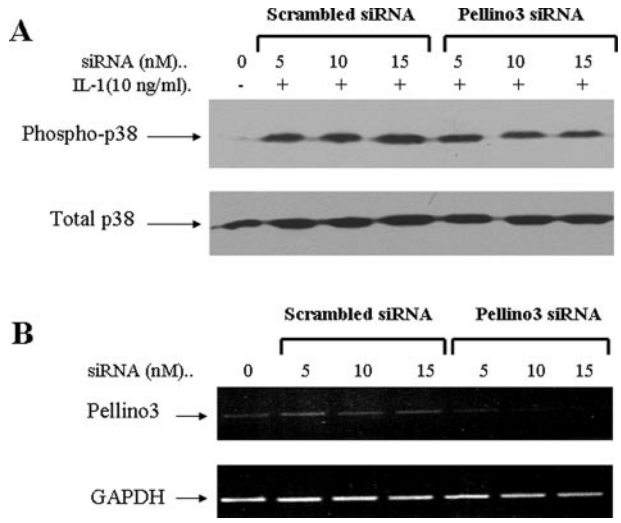


FIG. 2. **Pellino3 is involved in IL-1 activation of p38.** HEK293 cells were transfected in the absence or presence of various concentrations of siRNA specific for Pellino3 or a scrambled control siRNA (5–15 nM). Cells were treated 30 h post transfection with or without IL-1 (10 ng/ml) for 20 min. *A*, cell lysates were generated, subjected to polyacrylamide gel electrophoresis, and subsequently to Western immunoblotting. Blots were first probed with phospho-specific p38 antibodies and then stripped and re-probed using total p38 antibodies. Immunoreactivity was visualized by enhanced chemiluminescence. *B*, total cellular RNA was prepared and subjected to RT- PCR using primers to selectively amplify regions of the Pellino3 and GAPDH cDNAs. This resulted in the generation of products with the predicted sizes of 650 and 400 bp, respectively.

Confocal Microscopy—HEK293 cells (1.6×10^5/well) were seeded into 4-well Lab-Tek chamber slides (Nunc A/S, DK-4000, Denmark) and grown for 24 h. 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 antifade 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. 1*A*). 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. 1*A*) 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. 1*A*, *inset*).

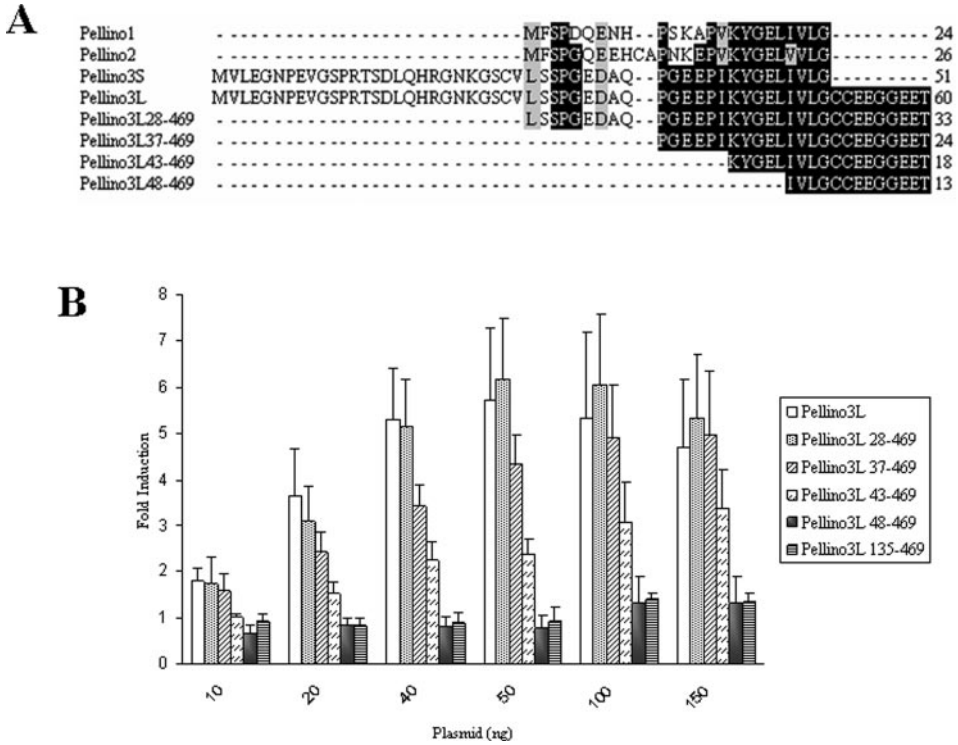


FIG. 3. **Mapping studies identify regions important for Pellino3 activation of p38 MAPK.** *A*, the alignment of the N-terminal regions of Pellino1, -2, and -3 and the initiation sites for Pellino3L truncation mutants are indicated (Pellino3L135–469 not presented). Sequence alignments were constructed using ClustalW and displayed using BOXSHADE. *B*, 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 and truncation mutants of 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 five independent experiments.

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. 1*B*). 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 phospho-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. 1*C*). 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.

Pellino3 Mediates IL-1 Activation of p38 MAPK—To address the physiological role of Pellino3 in the p38 MAPK pathway, siRNA that specifically targets Pellino3 was assessed for its effects on the IL-1-induced phosphorylation of endogenous p38 MAPK in HEK293 cells. Pellino3-specific siRNA caused a considerable reduction in the phosphorylation of p38 MAPK by IL-1, relative to a control Pellino3-scrambled siRNA (Fig. 2*A*). 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. 2*B*). 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.

Deletion Analysis of Pellino3 Reveals Sites Important for p38 MAPK Activation—We next attempted to identify 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. 3*A*). Furthermore, 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 using the CHOP *trans*-Reporting System. The truncation of the first 27 amino acids failed to affect the ability of Pellino3 to activate p38 MAPK, because both full-length Pellino3 and mutant 28–469 showed comparable patterns of dose-dependent activation in the assay (with both demonstrating maximum induction of 6-fold) (Fig. 3*B*). The next 9 and 15 amino acids were additionally truncated, because these regions show sequence diver-

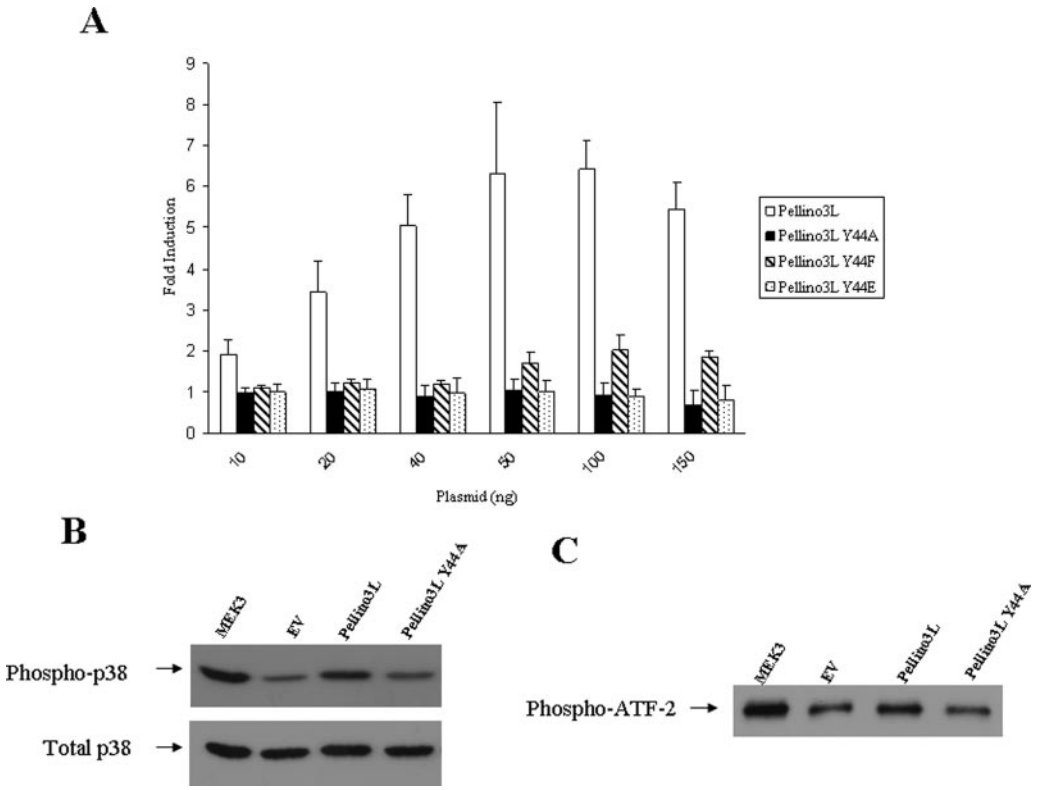


FIG. 4. **Mutational analysis of tyrosine residue in the N terminus of Pellino3 abrogates its ability to activate p38 MAPK.** *A*, HEK293 cells were co-transfected with the *trans*-activator plasmid pFA-CHOP, pFR-Luc (encoding GAL4-CHOP regulated firefly luciferase), phRL-TK (constitutively expressed *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 subjected to Western immunoblotting using anti-phospho-ATF-2 monoclonal antibody.

gence between Pellino3 and the other members of the family. These truncation mutants, 37–469 and 43–469, indeed showed reduced efficacy in activating p38 (5- and 3.5-fold, respectively, compared with 6-fold with wild-type construct) indicating that sites important for augmenting p38 MAPK activity lie within this stretch of 15 amino acids (Fig. 3*B*). However, the removal of the next 5 amino acids obliterated the ability of Pellino3 to activate p38 MAPK as evidenced by the complete ineffectiveness of mutants 48–469 and 135–469 in the assay (Fig. 3*B*). The inability of these mutants to activate p38 MAPK is not due to lack of expression (see below in Fig. 5).

The results of these transfection studies define a critical region (residues 43–47; KYGEL) in Pellino3 for manifesting activation of p38 MAPK. We next performed site-directed mutagenesis to identify specific individual residues in this region that are key to manifesting the activity. We initially mutated the lysine to alanine and this mutant was fully active in the CHOP transfection assay (data not shown). However the mutation of the immediately adjacent tyrosine residue (Tyr-44) in Pellino3L abolished its ability to activate p38 MAPK (Fig. 4*A*). This loss in activity was independent of the type of mutation, because mutation to phenylalanine or alanine leads to similar obliteration of activity. The crucial importance of Tyr-44 with respect to Pellino3 activation of p38 MAPK was further confirmed by demonstrating that mutation of this residue abolishes the ability of Pellino3 to induce phosphorylation of endogenous p38 MAPK (Fig. 4*B*) and thus leaves Pellino3 incapable of activating p38 MAPK to phosphorylate substrates such as ATF-2 (Fig. 4*C*). In an effort to explore the potential regulation of Pellino3 activity by phosphorylation of Tyr-44 we mutated the latter to the phospho-mimetic residue glutamic acid. However, the resulting mutant was inactive (Fig. 4*A*) questioning the importance of phosphorylation of this residue for activation of Pellino3.

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. 5*A*) and TAK-1 (Fig. 5*B*). 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. 5*C*). 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

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.

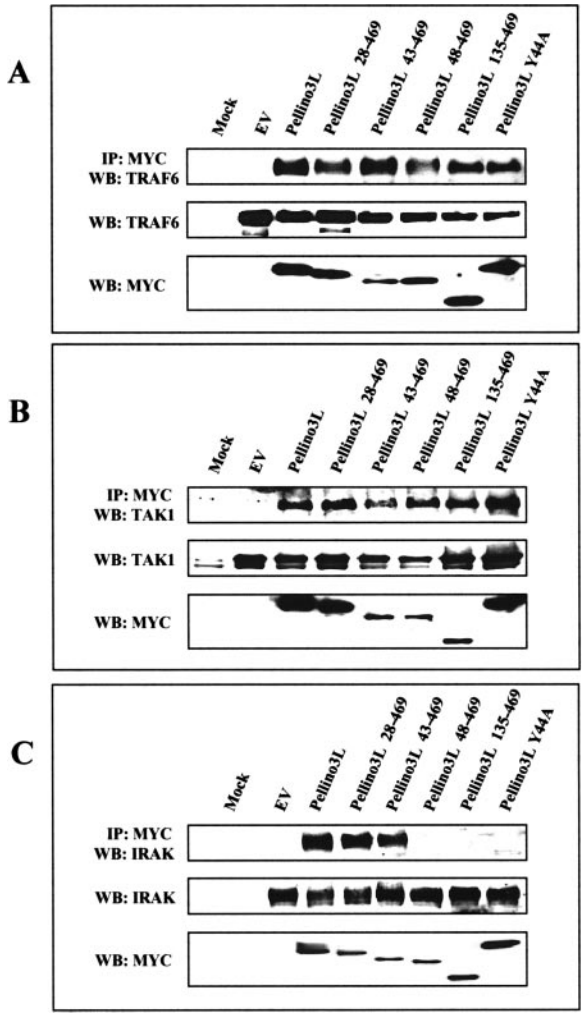
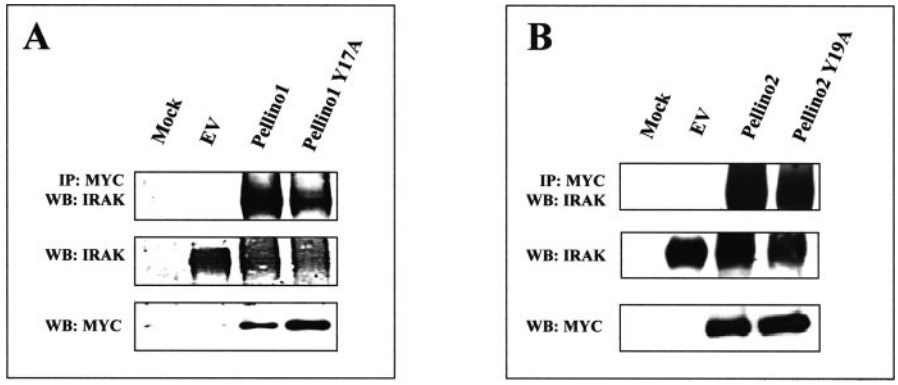


FIG. 5. **Mapping regions in Pellino3 that facilitate interaction with TRAF-6, TAK-1, and IRAK.** HEK293 cells were co-transfected with the designated *myc*-tagged Pellino constructs or the pTARGET empty vector (*EV*) and TRAF-6 (*A*) or TAK-1 (*B*) or IRAK (*C*) expression construct. Control cells were mock transfected (*Mock*) in the presence of pTARGET alone. Cell lysates were generated and immunoprecipitated with an immobilized anti-*myc* antibody. Immunoprecipitates were subjected to Western immunoblotting using the appropriate antibody against TRAF-6 (*A*) or TAK-1 (*B*) or IRAK (*C*). Cell lysates were also analyzed by Western immunoblotting to confirm expression of the constructs.

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. HEK293 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. 8*A*, *lower left panel*) as evidenced by co-localization with the DAPI-stained nuclei (Fig. 8*A*, *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. 8*B*, *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. 8*B*, *lower right panel*) due to the combined green and red fluorescence of Pellino3-pEGFP and MAPKAP kinase 2-DsRed, respectively.

FIG. 6. **Mutation of conserved tyrosine residue in the N termini of Pellino1 (*A*) and Pellino2 (*B*) fails to affect their interaction with IRAK.** HEK293 cells were co-transfected with the designated *myc*-tagged Pellino constructs or the pTARGET empty vector (*EV*) and IRAK expression construct. Control cells were mock transfected (*Mock*) in the presence of pTARGET alone. Cell lysates were generated and immunoprecipitated with an immobilized anti-*myc* antibody. Immunoprecipitates were subjected to Western immunoblotting using an anti-IRAK antibody. Cell lysates were also analyzed by Western immunoblotting to confirm expression of the constructs.



Interestingly, Pellino3-pEGFP showed intense expression in as yet undefined structure(s) in the cytoplasm of the cell (Fig. 8*B*, *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. 8*C*, *lower left panel*). The lack of significant redistribution to the cytoplasm is evidenced by the absence of *yellow fluorescence* in the montage (Fig. 8*C*, *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. 8*C*,

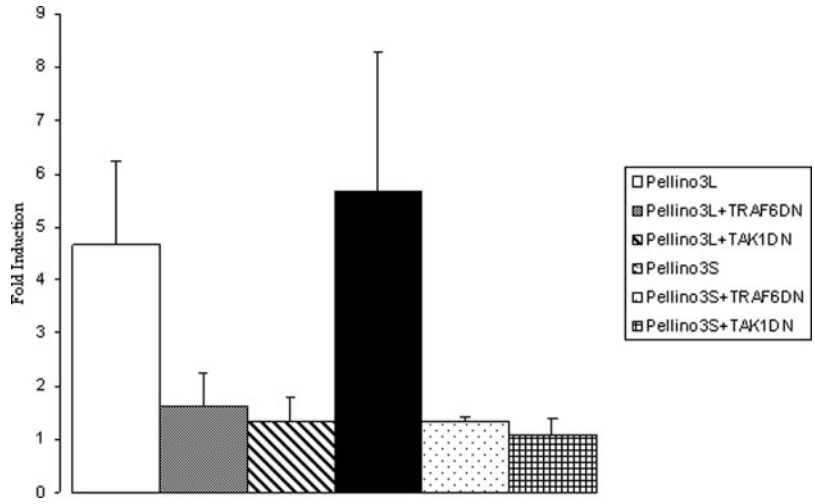


FIG. 7. **Pellino3 activates p38 MAPK via TRAF-6 and TAK-1.** HEK293 cells were co-transfected with the *trans*-activator plasmid pFA-CHOP, pFR-Luc (encoding GAL4-CHOP regulated firefly luciferase), phRL-TK (constitutively expressed *Renilla* luciferase), and plasmids encoding Pellino3L or Pellino3S (50 ng) in the presence or absence of dominant negative TRAF-6 or TAK-1 constructs (100 ng). 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 four independent experiments.

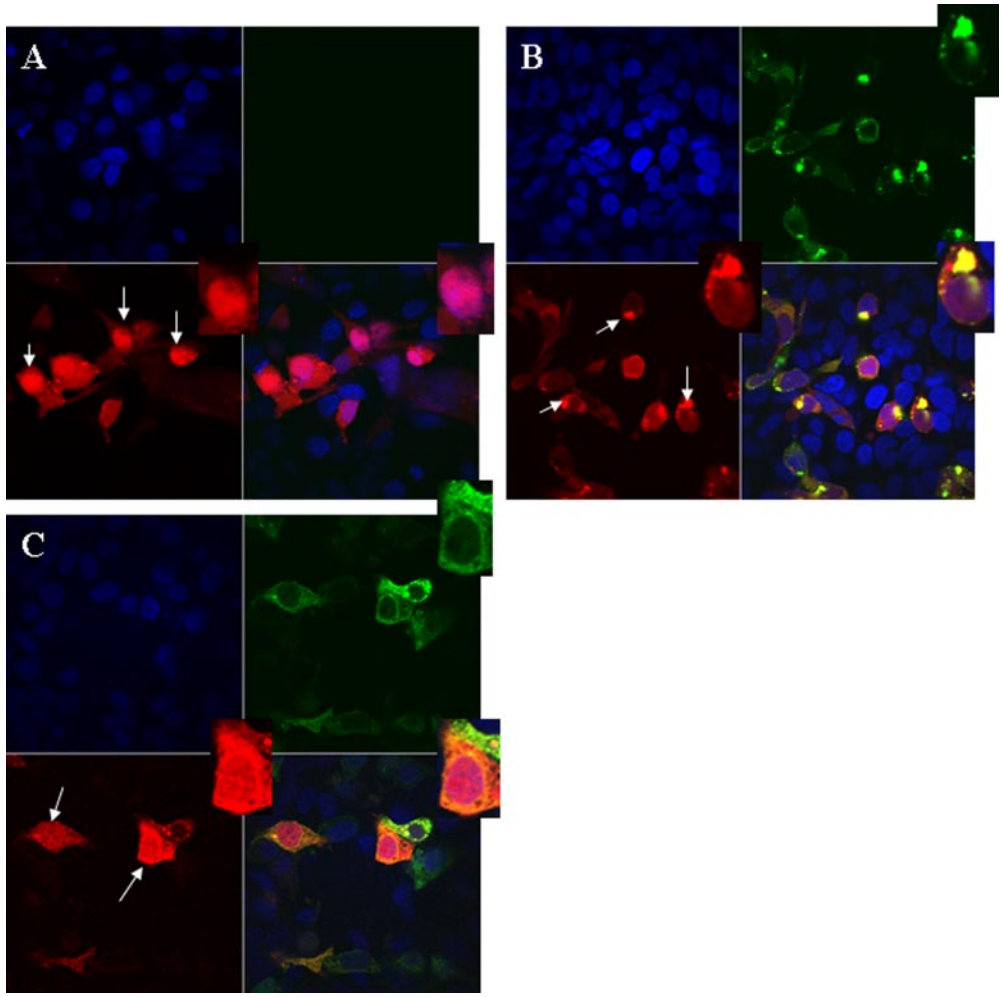


FIG. 8. **Pellino3 promotes the nuclear export of MAPKAP kinase 2.** HEK293 cells were co-transfected with MAPKAP kinase 2-DsRed and pTARGET empty vector (*A*), Pellino3-pEGFP (*B*), or Pellino3 (Y44A)-pEGFP (*C*). Confocal images were captured using the ×63 objective (oil immersion) on the UV Zeiss 510 Meta System laser scanning microscope equipped with the appropriate filter sets and analyzed using the LSM 5 browser imaging software. The *upper left panels* indicate DAPI staining of the nuclei. The *upper right panels* represent images of isolated pEGFP fluorescence. The *lower left panels* indicate images of isolated MAPKAP kinase 2-DsRed fluorescence. The l*ower right panels* are montages of the three other images.

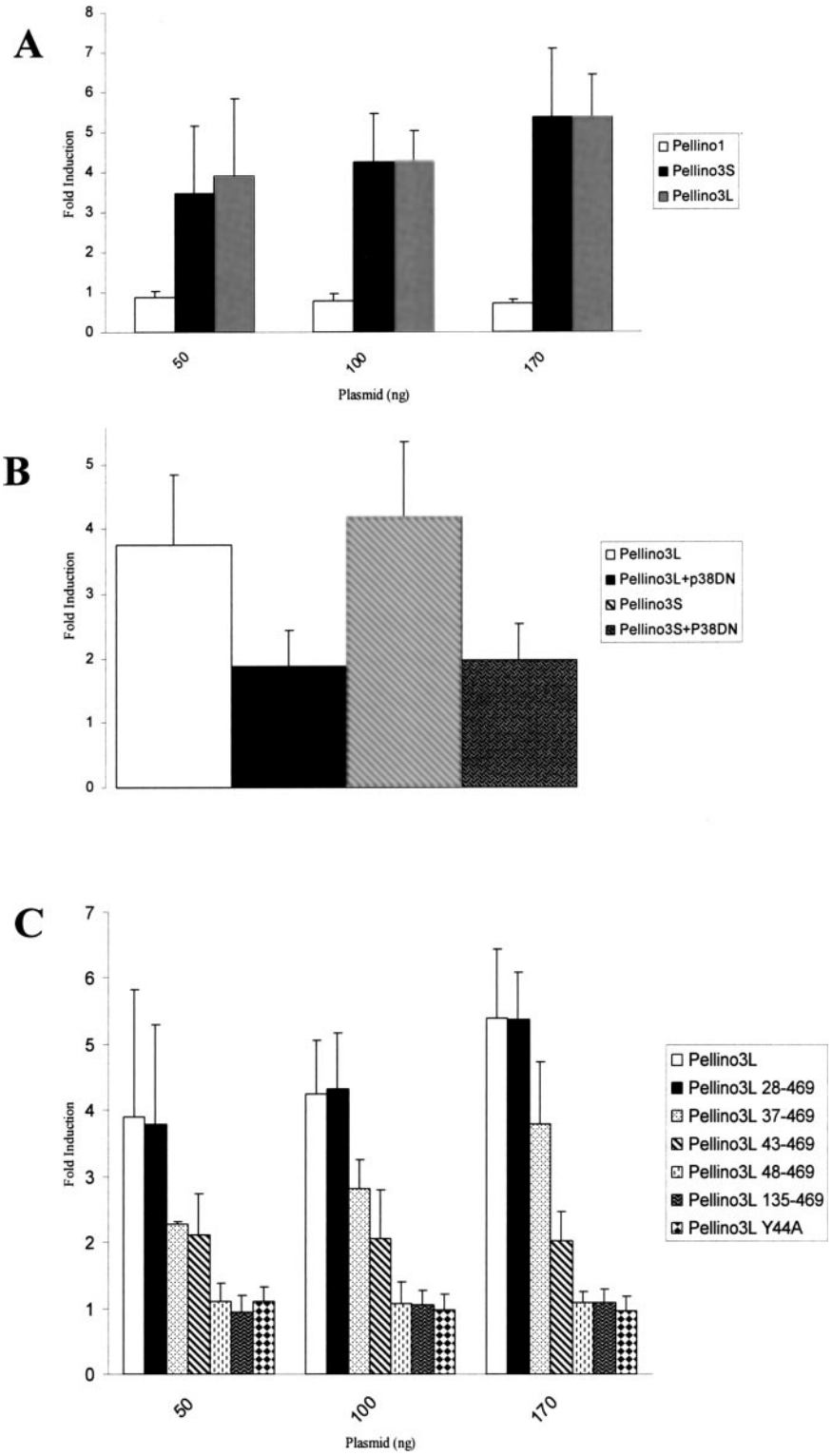


FIG. 9. **Pellino3 activates CREB in a p38 MAPK dependent manner.** HEK293 cells were co-transfected with a CREB-regulated firefly luciferase reporter, phRL-TK (constitutively expressed *Renilla* luciferase), and the following expression plasmids: *A*, Pellino1, Pellino3L, or Pellino3S; *B*, Pellino3L or Pellino3S (100 ng) with or without a construct encoding dominant negative p38 (p38DN) (70 ng); *C*, Pellino3L or its designated mutants. Cell extracts were generated the following day and assayed for firefly (a measure of CREB transactivation) and *Renilla* (for normalizing transfection efficiency) luciferase. Data are presented relative to cells transfected with pCMV5 empty vector alone. Results represent mean ± S.E. of four independent experiments.

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. 9*A*). 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. 9*B*). 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. 9*C*). 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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Pellino3 Is a Novel Upstream Regulator of p38 MAPK and Activates CREB in a p38-dependent Manner

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