Kinase-active Interleukin-1 Receptor-associated Kinases Promote Polyubiquitination and Degradation of the Pellino Family

DIRECT EVIDENCE FOR PELLINO PROTEINS BEING UBQUITIN-PROTEIN ISOPÉPTIDE LIGASES

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Members of the Pellino family are interleukin-1 receptor-associated kinase (IRAK)-interacting proteins that possess RING-like domains. The presence of these domains led to the suggestion that Pellino proteins are ubiquitin-protein isopeptide ligases (E3). However, no conclusive data currently exist to prove this proposal. This study provides the first direct evidence that Pellino proteins possess E3 activity. Recombinant forms of Pellino1 and Pellino2 and both spliced variants of Pellino3 are shown in an in vitro ubiquitination assay to be E3 ligases that catalyze Lys63-linked polyubiquitination, with Pellino3 exhibiting the greatest ligase activity. Whereas the Pellino proteins cause polyubiquitination of IRAK-1, we also show that kinase-active members of the IRAK family (IRAK-1 and IRAK-4) promote reciprocal polyubiquitination of the Pellino proteins and that this is associated with IRAK-induced degradation of the Pellino family. In contrast, IRAK-2 (which lacks a functional kinase domain) and kinase-dead forms of IRAK-1 and IRAK-4 fail to degrade the Pellino proteins. We show that these kinase-inactive IRAK proteins can associate with Pellino proteins, thus excluding the possibility that their inability to regulate Pellino degradation is due to lack of association with the Pellino proteins. The physiological relevance of IRAK-induced degradation of Pellino proteins is confirmed by the demonstration that lipopolysaccharide causes degradation of endogenous forms of Pellino3 in peripheral blood mononuclear cells. In summary, this study not only demonstrates Pellino proteins to be E3 ligases that can catalyze Lys63-linked polyubiquitination but also shows bidirectional signaling between the IRAK and Pellino families and highlights a novel function for IRAK kinase activity.

Toll-like receptors (TLRs)3 detect and respond to conserved microbial structures or products of microbial metabolism known as pathogen-associated molecular patterns (1). The signaling pathways triggered by TLRs share much in common with those activated by the interleukin-1 (IL-1) receptor due to the presence of a conserved intracellular Toll/IL-1 receptor domain. Following ligand stimulation, TLRs/IL-1 receptors associate with Toll/IL-1 receptor domain-containing adaptor proteins such as MyD88, leading to the recruitment of members of the IL-1 receptor-associated kinase (IRAK) family of serine/threonine kinases (2). IRAK-4 initially phosphorylates IRAK-1, thus stimulating the kinase activity and intensive autophosphorylation of IRAK-1 (3, 4). Hyperphosphorylated IRAK-1 interacts with the downstream signaling molecule TRAF-6, resulting in their coordinated dissociation from the receptor complex and interaction at the plasma membrane with the MAP3 kinase TAK1 and associated proteins TAB1 and TAB2 (5). TAK1 becomes phosphorylated, and this leads to dissociation of a TRAF-6-TAK1-TAB1-TAB2 complex from IRAK-1 in the membrane (6). The complex moves to the cytosol, where TRAF-6, a RING domain-containing ubiquitin-protein isopeptide ligase (E3), interacts with the ubiquitin carrier protein (E2)-conjugating heterodimer UbcH13/Uev1a, resulting in Lys63 polyubiquitination of TRAF-6 and subsequent activation of TAK1 (7). Whereas ubiquitination by Lys48-linked ubiquitin polymers targets proteins for ATP-dependent proteolysis by the proteasome (8), Lys63-linked ubiquitin chains mediate non-classical, degradation-independent modifications (9) such as activation of TRAF-6. Activated TAK1 stimulates IxB kinases, which trigger transcription factor NF-κB and MKK3/6 and MKK4, which activate the MAPKs p38 and JNK, respectively (10).

Members of the IRAK family are key mediators in TLR/IL-1 receptor (IL-1R) signaling pathways. The family consists of four members: IRAK-1 (11), IRAK-2 (12), IRAK-M (13), and IRAK-4 (4). IRAK-4 appears to be the most critical, as TLR/IL-1R signaling is severely impaired in cells lacking this protein (14) compared with a partial impairment in IRAK-1-deficient cells (15). In contrast, IRAK-M functions as a negative regulator of TLR signaling (16). The physiological role of IRAK-2 is unknown. Both IRAK-1 and IRAK-4 have intrinsic kinase activity, whereas IRAK-2 and IRAK-M are catalytically inactive (13). Although the kinase activity of IRAK-4 is essential for some aspects of TLR/IL-1R signaling (4, 17, 18), the kinase activity of IRAK-1 is dispensable for its role in these signaling pathways (19–21), and thus, the functional relevance of IRAK-1 kinase activity remains to be elucidated.
Both IRAK-1 and IRAK-4 interact with the Pellino family of proteins. Pellino1 interacts with IRAK, IRAK-4, TRAF-6, and TAK1 and is required for activation of NF-κB (22) but is not involved in activation of the MAPK pathways (23). Pellino2 also interacts with these same proteins (24, 25), but there are contrasting reports on its role in activation of NF-κB and the MAPKs JNK and ERK (23–25). Both spliced forms of Pellino3 interact with IRAK, TRAF-6, and TAK1 and activate JNK, ERK, and p38 MAPK pathways (26, 27). However, a clear mechanistic understanding of the roles and regulation of Pellino proteins in TLR/IL-1R signaling pathways has yet to emerge. The interaction with IRAK-1 and IRAK-4 is likely to be of key importance because both forms are capable of promoting phosphorylation of the Pellino proteins (25, 28). The consequence of this phosphorylation is unknown. Furthermore, overexpression of Pellino1 and Pellino2 can promote polyubiquitination of IRAK-1, and given that Pellino proteins contain a RING-like domain, a role for Pellino proteins as E3 ligases has been proposed (28). However, the overexpression approach does not allow distinguishing between a direct or indirect mode of action, and no evidence currently exists to definitively show that members of the Pellino family have E3 activity. Furthermore, the type of lysine linkage in the ubiquitination process that is promoted by Pellino proteins is unknown.

We now provide the first direct evidence that Pellino proteins are E3 ligases that catalyze Lys^{63}-linked polyubiquitination. We also demonstrate that IRAK/Pellino interactions lead to polyubiquitination of both binding partners. Furthermore, IRAK-1 and IRAK-4 promote polyubiquitination and degradation of the Pellino proteins in a kinase-dependent manner. Although the E3 activity of Pellino proteins mediates Pellino-induced polyubiquitination of IRAK-1, Pellino proteins are substrates for other E3 ligases. This study provides a major advance in our mechanistic understanding of the functional consequences of IRAK/Pellino interactions.

**MATERIALS AND METHODS**

**Biological Reagents and Expression Constructs**—Anti-IRAK-1, anti-ubiquitin, and anti-green fluorescent protein antibodies were from Santa Cruz Biotechnology, Inc. Anti-Myc monoclonal antibody was from Cell Signaling Technology. Anti-FLAG and anti-β-actin antibodies were from Sigma. Recombinant forms of ubiquitin (wild-type, K48R, and K63R), ubiquitin-activating enzyme (E1), and E2 (UbcH13/Uev1a) were from Boston Biochem. Rosetta cells (Escherichia coli) were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% (v/v) fetal bovine serum, 100 µg/ml penicillin G, and 100 µg/ml streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂. Cells were passaged using 1% (w/v) trypsin in phosphate-buffered saline. Human peripheral blood mononuclear cells (PBMCs) were isolated from blood taken from healthy donors using Lymphoprep according to the manufacturer’s instructions. PBMCs were cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 µg/ml penicillin G, and 100 µg/ml streptomycin. Ligand stimulation was performed on cells in serum-containing medium at 37 °C for all experiments.

**Generation of Recombinant Pellino Proteins and in Vitro Ubiquitination Assay**—Myc-tagged Pellino3L, Pellino3S, Pellino2, and Pellino1 were cloned into the His-tagged vector pRSET-A. Escherichia coli cells (Rosetta cells) were transformed with the constructs and grown for 24 h in Terrific broth medium (Overnight Express). Cultures were centrifuged, and the pellet was resuspended in BugBuster protein extraction reagent as recommended by the manufacturer. Protein purification was carried out using the His-Bind purification kit following the manufacturer’s instructions. The purity and integrity of recombinant proteins were confirmed by subjecting samples to SD-page and PAGE (followed by Coomassie staining) as well as to Western immunoblotting using anti-Myc antibody. For the in vitro ubiquitination assay, recombinant Pellino protein (1 µg) was incubated with ubiquitin (wild-type, K48R, and K63R; 2 µg), E1 (50 ng), UbcH13/Uev1a (400 ng), and protease inhibitor mixture (EDTA-free) in 5 mM Tris–HCl (pH 7.5) containing 2 mM MgCl₂, 2 mM ATP, and 100 mM NaCl. Reactions were incubated at 37 °C for 2 h and terminated by the addition of SDS-PAGE sample buffer. Samples were boiled for 5 min, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting using anti-ubiquitin antibody.

**Transfection of Cells**—Cells were seeded at 2 × 10⁵/ml and 2.5 × 10⁶/ml in 6-well plates (3 ml/well; for immunoprecipitation analysis) and in 12-well plates (1.5 ml/well; for western blot analysis), respectively, and grown for 24 h to ~80% confluency. For each well of a 6-well plate, 4 µg of total DNA (consisting of the indicated amounts of expression constructs) was added to Opti-MEM 1 (250 µl), and Lipofectamine (8 µl) was also diluted in Opti-MEM 1 (250 µl). The DNA and Lipofectamine solutions were incubated separately at room temperature for 5 min. For each well of a 12-well plate, 1.6 µg of total DNA was added to Opti-MEM 1 (100 µl), and Lipofectamine (2.5 µl) was diluted in Opti-MEM 1 (100 µl). After the 5-min incubation, the DNA and Lipofectamine solutions were mixed gently and incubated at room temperature for 20 min. 1 or 0.5 ml of the medium was removed from each well prior to the
addition of 500 or 200 μl of Lipofectamine-DNA complexes to each well of the 6- or 12-well plate, respectively.

**Immunoprecipitation and Western Blot Analysis**—Transfected cells were incubated at 37 °C for 24 h. The medium was then removed, and cells were washed once with ice-cold phosphate-buffered saline. An aliquot (500 μl) of lysis buffer (50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 0.5% (v/v) Igepal, 50 mM NaF, 1 mM Na3VO4, 1 mM dithiothreitol, 1 mM phenyl-methylsulfonyl fluoride, and protease inhibitor mixture (25 μg/ml leupeptin, 25 μg/ml apro tin, 1 mM benzamidine, and 10 μg/ml trypsin inhibitor) was added to each well and incubated at 4 °C for 30 min with gentle rocking. Cell lysates were centrifuged at 12,000 × g for 10 min. An aliquot (50 μl) of the supernatant was retained for Western blot analysis, and the remainder was subjected to immunoprecipitation.

Cell lysates were initially precleared by the addition of anti-IgG antibody (1 μg) and resuspended protein A/G-agarose (10 μl) to each sample. Incubations were maintained at 4 °C with rocking for 30 min. The agarose matrix was pelleted by centrifugation at 10,000 × g for 5 min at 4 °C. The supernatant was removed to a fresh microcentrifuge tube. The appropriate anti-methylsulfonyl fluoride, and protease inhibitor mixture (25 μg/ml leupeptin, 25 μg/ml apro tin, 1 mM benzamidine, and 10 μg/ml trypsin inhibitor) was added to each well and incubated at 4 °C for an additional 24 h at 4 °C. Immunoprecipitates were collected by passing the samples through Cytosignal filters by centrifugation at 16,000 × g for 10 min. An aliquot (50 μl) of the supernatant was retained for Western blot analysis, and the remainder was subjected to immunoprecipitation.

Cell lysates were initially precleared by the addition of anti-IgG antibody (1 μg) and resuspended protein A/G-agarose (10 μl) to each sample. Incubations were maintained at 4 °C with rocking for 30 min. The agarose matrix was pelleted by centrifugation at 10,000 × g for 5 min at 4 °C. The supernatant was removed to a fresh microcentrifuge tube. The appropriate anti-body was added (2 μg/sample) and incubated overnight at 4 °C with rocking. An aliquot (35 μl) of resuspended protein A/G-agarose was added to each sample and incubated with rocking for an additional 24 h at 4 °C. Immunoprecipitates were collected by passing the samples through Cytosignal filters by centrifugation at 16,000 × g for 1 min. The beads were then washed four times with 500 μl of lysis buffer (without Na3VO4, dithiothreitol, phenylmethylsulfonyl fluoride, or protease inhibitor mixture). An aliquot (60 μl) of SDS-PAGE sample buffer was added to the trapped agarose, and the immunoprecipitated material was eluted by centrifugation at 16,000 × g for 1 min. Samples were boiled for 5 min, resolved by SDS-PAGE, transferred to nitrocellulose membranes, and analyzed by immunoblotting using the appropriate antibodies. Immunoreactivity was visualized by enhanced chemiluminescence.

**Analysis of Endogenous Pellino3**—To assay for the E3 activity of endogenous forms of Pellino, Pellino3 was immunoprecipitated from HEK293 cells as described above using rabbit anti-Pellino3 polyclonal antibody. The immunoprecipitate was then incubated with E1 and E2 in the in vitro ubiquitination assay and assayed as described above.

For expression analysis of Pellino3 in PBMCs, cells were seeded at 1.5 × 10⁶/ml in 6-well plates (2 ml/well) and left overnight. Cells were then stimulated with lipopolysaccharide (LPS; 1 μg/ml) or tumor necrosis factor (TNF; 100 ng/ml) for the indicated times, harvested in SDS-PAGE sample buffer, boiled, and resolved by 10% SDS-PAGE. Separated proteins were transferred onto nitrocellulose membranes and subjected to immunoblotting using rabbit anti-Pellino3 or anti-β-actin polyclonal antibody. Immunoreactivity was visualized by enhanced chemiluminescence.

**RESULTS**

**Pellino Proteins Are E3 Ligases That Catalyze Lys⁶³-linked Ubiquitination**—Although Pellino proteins have been shown to promote ubiquitination of IRAK-1, this process may be direct or indirect, and no evidence currently exists to definitively show that Pellino proteins possess E3 activity. Furthermore, irrespective of the mode of action, no studies have explored the type of lysine linkage that is catalyzed by Pellino proteins. To address these issues, we generated recombinant forms of each of the Pellino proteins and assayed for potential E3 activity in an in vitro ubiquitination assay. Each of the Pellino proteins were incubated with recombinant wild-type ubiquitin in the presence and absence of E1 and the E2 heterodimer UbcH13/Uev1a. Reactions were then subjected to SDS-PAGE followed by Western blot analysis using anti-ubiquitin antibody. Polyubiquitination, as evidenced by an immunoreactive ubiquitin smear, was only apparent when E1 and E2 were incubated with members of the Pellino family (Fig. 1A, left panel). All three Pellino proteins, including both spliced forms of Pellino3 (Fig. 1B), catalyzed polyubiquitination, providing the first direct evidence that Pellino proteins are E3 ligases. Pellino3 showed especially strong activity, with Pellino1 and Pellino2 being somewhat weaker.

We next investigated the type of lysine linkage that Pellino proteins employ in catalyzing polyubiquitination. To this end, the same in vitro assays were performed but using recombinant mutant forms of ubiquitin with Lys⁴⁸ mutated to arginine (K48R) or Lys⁵³ mutated to arginine (K63R). With K48R ubiquitin used as the substrate, each of the Pellino proteins retained their capacity to catalyze polyubiquitination (Fig. 1, middle panels), indicating that Pellino-induced polyubiquitination in the presence of UbcH13/Uev1a is not mediated by Lys⁴⁸-linked chains. However, each of the Pellino proteins lost all ability to catalyze polyubiquitination when K63R ubiquitin was used as the substrate (Fig. 1, right panels), thus showing for the first time that Pellino proteins are E3 ligases that catalyze Lys⁶³-linked ubiquitination.

To confirm that E3 activity is a function of endogenous Pellino proteins in cells, we aimed to immunoprecipitate endogenous forms of Pellino and to assay their E3 activity in the in vitro ubiquitination assay. To date, there has been a lack of antibodies that recognize endogenous forms of the Pellino proteins. We thus attempted to raise antibodies against the Pellino proteins and were successful in generating a rabbit polyclonal antibody that recognizes Pellino3. This antibody was used to immunoprecipitate Pellino3 from HEK293 cell extracts, and the immunoprecipitate was incubated with wild-type ubiquitin in the presence of E1 and the E2 heterodimer UbcH13/Uev1a. Western blot analysis with anti-ubiquitin antibody demonstrated strong polyubiquitination as evidenced by the immunoreactive ubiquitin smear (Fig. 1C). In contrast, an immunoprecipitate that was generated using anti-Myc antibody failed to show any E3 activity, confirming the specificity of the activity for immunoprecipitated Pellino3. Such data emphasize that the E3 activity of Pellino proteins is of physiological relevance.

**IRAK-1 and IRAK-4 Promote Polyubiquitination of Pellino Proteins in a Kinase-dependent Manner**—Given that Pellino proteins are subject to phosphorylation by IRAK-1 and that autoubiquitination is a common feature of E3 ligases, we next addressed whether IRAK-1 can regulate ubiquitination of Pellino proteins in a kinase-dependent manner. Cells were transfected with constructs encoding tagged members of the Pellino family in the absence and presence of plasmids expressing wild-
type and kinase-dead forms of IRAK-1. Pellino proteins were subsequently immunoprecipitated and probed by Western blotting for ubiquitination using anti-ubiquitin antibody. When transfected in the absence of IRAK constructs, each of the wild-type Pellino proteins showed a predominant single band with mobility consistent with monoubiquitination (Fig. 2, upper left panel). Two faint bands of slower mobility were also apparent in each sample, suggestive of low levels of polyubiquitination. However, the coexpression of wild-type IRAK-1 stimulated intensive polyubiquitination of each of the Pellino proteins as manifested by smears of immunoreactive ubiquitin. The IRAK-induced polyubiquitination was especially strong in the case of the two spliced forms of Pellino3. Some of the slower moving region of the ubiquitin smear is likely to represent polyubiquitination of coprecipitated IRAK-1. Indeed, Western blot analysis of these samples using anti-IRAK-1 antibody clearly demonstrated that coexpression of each of the wild-type Pellino proteins with IRAK-1 resulted in slower migrating forms of IRAK-1, and this was visualized as a smear above the banding normally observed with IRAK-1 alone (Fig. 2, middle panels). It is worth emphasizing that both spliced forms of Pellino3 caused polyubiquitination of IRAK-1 and that this contrasts with a previous report suggesting that Pellino3 is incapable of regulating ubiquitination of IRAK-1 (28).

The present data show that the interaction of a Pellino protein and IRAK-1 leads to polyubiquitination of both binding partners. Such reciprocal polyubiquitination is dependent on Pellino/IRAK-1 complex formation because no polyubiquitination was observed when Pellino3L Y44A, a point mutant that fails to interact with IRAK-1, was coexpressed with IRAK-1 (Fig. 2). Furthermore, polyubiquitination of Pellino proteins and IRAK-1 is dependent on the kinase activity of IRAK-1. The coexpression of kinase-dead IRAK-1 (unlike that of its wild-type counterpart) with members of the Pellino family failed to manifest any sign of polyubiquitination of itself or the Pellino proteins (Fig. 2, right panels). Consequently, only the basal monoubiquitination of Pellino proteins was detected when coexpressed with kinase-dead IRAK-1. These results strongly suggest that polyubiquitination of Pellino proteins is a functional consequence of IRAK-induced phosphorylation of these proteins.
Because IRAK-4 is a known upstream regulator of IRAK-1 by stimulating the kinase activity of the latter, we next investigated whether IRAK-4 can also promote polyubiquitination of Pellino proteins. The coexpression of IRAK-4 (similar to that of IRAK-1) with each of the Pellino constructs induced polyubiquitination of Pellino proteins in a kinase-dependent manner. However, IRAK-4 promoted major polyubiquitination of both spliced forms of Pellino3 and much weaker polyubiquitination of Pellino1 and Pellino2 (Fig. 3, upper panels). To investigate a possible basis for the varying degrees of polyubiquitination of the different Pellino proteins, immunoprecipitated IRAK-4 was probed for levels of coprecipitating Pellino proteins. Although IRAK-4 was shown to associate with all members of the Pellino family, it was most effective in coprecipitating the two forms of Pellino3 (Fig. 3, middle panels). This suggests that Pellino3 interacts more strongly than Pellino1 or Pellino2 with IRAK-4, and this likely explains the very prominent polyubiquitination of Pellino3 by IRAK-4.

Mutation of the RING-like Domain in Pellino3 Abrogates Polyubiquitination of IRAK-1 but Fails to Affect IRAK-1-induced Polyubiquitination of Pellino3—We next addressed Pellino autoubiquitination as a potential mechanism that mediates IRAK-1-induced polyubiquitination of Pellino proteins. Using Pellino3L as a model, we mutated its RING-like domain and assessed the susceptibility of the mutant to IRAK-1-induced polyubiquitination. The RING-like domain of Pellino proteins has a CHC2CHC2 motif that differs slightly from the classical C3HC4 RING motif. A mutant of Pellino3L was generated (Pellino3L C384A/C387A) and represents a double point mutation of both cysteine residues in the first C2 motif. Like its wild-type counterpart, the Pellino3L C384A/C387A mutant was susceptible to modification by kinase-active IRAK (Fig. 4A). In the presence of IRAK-1, the mutant migrated as a smear, consistent with modification by ubiquitination. This was confirmed by Western blot analysis of immunoprecipitated Pellino3L C384A/C387A using anti-ubiquitin antibody (Fig. 4B). It should be noted that the immunoprecipitated RING mutant of Pellino3L displayed reduced polyubiquitination relative to its wild-type counterpart. This was apparent only in the slower migrating region of the ubiquitin smear and likely represents reduced polyubiquitination of coprecipitated IRAK-1 because the latter migrated with a mobility corresponding to this region (Fig. 4B, middle panel). Thus, the E3 activity of Pellino3L is dispensable for its own IRAK-1-induced polyubiquitination but is important for mediating polyubiquitination of IRAK-1.

**IRAks Stimulate Pellino Polyubiquitination/Degradation**

**IRAk-1 and IRAK-4 Induce the Degradation of Pellino Proteins in a Kinase-dependent Manner**—We next addressed the functional consequence of IRAK-1-induced polyubiquitination of the Pellino proteins. In our earlier IRAK/Pellino interaction studies, we noted that coexpression of IRAK-1 decreased the expression levels of the Pellino proteins. We probed this effect in more detail with a view to investigating whether IRAK-1-induced polyubiquitination of the Pellino proteins may lead to Pellino degradation. HEK293 cells were cotransfected with plasmids encoding each of the Myc-tagged Pellino proteins and wild-type IRAK-1 or a kinase-dead mutant of IRAK-1. Cell extracts were generated the following day, and expression of the Pellino constructs was examined by Western immunoblotting using an antibody that detects the Myc epitope tag. Overexpression of IRAK-1 was found to promote the degradation of each of the wild-type Pellino proteins (Fig. 5A). It is interesting that the catalytically inactive mutant of IRAK-1 did not reduce the expression levels of Pellino proteins, indicating that the kinase activity of IRAK-1 is necessary to induce their degradation. Despite contrasting reports on the requirement of the kinase activity of IRAK-1 for interacting with Pellino (25, 26), the ineffectiveness of kinase-dead IRAK-1 in reducing the expression levels of Pellino proteins in this study was not due to lack of interaction because kinase-dead IRAK-1 coprecipitated with Pellino proteins in immunoprecipitation analysis (Fig. 5B). As shown in the above polyubiquitination studies, IRAK-1-induced degradation of Pellino proteins is dependent on Pellino/IRAK-1 complex formation because no degradation was observed when Pellino3L Y44A, the point mutant that fails to interact with kinase-active IRAK-1, was coexpressed with IRAK-1 (Fig. 5A). This also
serves as a valuable control to validate the specificity of the effects of IRAK-1 on Pellino proteins.

Because IRAK-4 is a known upstream regulator of IRAK-1 by stimulating the kinase activity of IRAK-1, we next investigated whether IRAK-4 can also promote the degradation of the Pellino proteins. Overexpression of wild-type IRAK-4 (like that of IRAK-1) caused the degradation of all three Pellino proteins, whereas its kinase-dead mutant was without effect (Fig. 6A). Using HEK293 11A cells, which lack IRAK-1, we next assessed whether IRAK-4-induced degradation of Pellino proteins is mediated by IRAK-1. Kinase-active IRAK-4 was still capable of degrading the Pellino proteins in IRAK-1-deficient cells (Fig. 6A), suggesting that there is a functional redundancy between IRAK-1 and IRAK-4 in this respect. The ability of IRAK-4 to affect the expression levels of Pellino proteins independently of IRAK-1 is suggestive of direct IRAK-4/Pellino interactions. Indeed, we now show for the first time that IRAK-4 interacts with all three Pellino proteins, characterized by especially strong interactions with both spliced forms of Pellino3 (Fig. 6B). The interactions are independent of the kinase activity of IRAK-4 because kinase-dead IRAK-4 also interacted with the Pellino proteins. Thus, the lack of effect of kinase-dead IRAK-4 on Pellino expression levels is likely due to defective kinase activity and not lack of ability to associate with Pellino proteins. It is interesting that Pellino3L Y44A, which fails to interact with IRAK-1, also lacked the ability to associate with IRAK-4 (Fig. 6B), and its expression levels were unaffected by kinase-active IRAK-4 (Fig. 6A).

IRAK-2 Interacts with the Pellino Proteins but Fails to Regulate Their Degradation —The findings that the kinase activity of IRAK-1 and IRAK-4 is necessary to induce the degradation of Pellino proteins prompted an investigation into the ability of a catalytically inactive IRAK family member to alter the expression levels of members of the Pellino family. IRAK-2 was chosen, as it contains an asparagine instead of an aspartate in its kinase domain, rendering the kinase domain inactive (12). IRAK-2 failed to affect the expression levels of any of the Pellino proteins (Fig. 7A) despite being able to interact with all members of the Pellino family (Fig. 7B). This suggests that IRAK-mediated degradation of Pellino proteins is restricted to members of the IRAK family that have an active kinase domain.

LPS Causes the Degradation of Pellino3 in Human PBMCs —The physiological relevance of IRAK-induced degradation of Pellino proteins was next investigated. Because LPS is a TLR-4 ligand that is known to employ IRAK-1 and IRAK-4, we examined the ability of LPS to regulate the degradation of endogenous Pellino. Human PBMCs were incubated with LPS for var-

**FIGURE 4. Mutation of the RING-like domain in Pellino3 abrogates polyubiquitination of IRAK-1 but fails to affect IRAK-1 induced polyubiquitination of Pellino3.** A, HEK293 cells were transfected with Myc-tagged Pellino3L or Pellino3L C384A/C387A (1 μg) in the presence or absence of an IRAK-1 expression construct (1 μg). Cell lysates were generated the following day and subjected to PAGE and subsequently to Western immunoblotting (WB) using anti-Myc antibody. B, HEK293 cells were cotransfected with Myc-tagged Pellino3L or Pellino3L C384A/C387A or the pcDNA3.1 empty vector (−; 3 μg) and an IRAK-1 expression construct (1 μg). Cell lysates were generated the following day and immunoprecipitated (IP) with anti-Myc antibody. Immunoprecipitates were subjected to PAGE and subsequently to Western immunoblotting using anti-ubiquitin (Ubiq; upper panel), anti-IRAK-1 (middle panel), and anti-Myc (lower panel) antibodies. Immunoreactivity was visualized by enhanced chemiluminescence. The mobilities of molecular mass markers are indicated in kilodaltons.

**FIGURE 5. IRAK-1 induces the degradation of Pellino proteins in a kinase-dependent manner.** A, HEK293 cells were cotransfected with plasmid encoding Myc-tagged Pellino3L, Pellino2, Pellino1, or Pellino3L Y44A (0.5 μg) and the pcDNA3.1 empty vector (−) or a wild-type (WT) or kinase-dead (KD) IRAK-1 construct (0.5 μg). Cell lysates were generated the following day and subjected to PAGE and subsequently to Western immunoblotting (WB) using anti-Myc antibody. B, HEK293 cells were cotransfected with the designated Myc-tagged Pellino constructs or the pcDNA3.1 empty vector (−; 3 μg) and a FLAG-tagged kinase-dead IRAK-1 expression construct (1 μg). Cell lysates were generated the following day and immunoprecipitated (IP) with anti-Myc antibody. Immunoprecipitates were subjected to PAGE and subsequently to Western immunoblotting using anti-IRAK-1 antibody. Cell lysates were also analyzed by Western immunoblotting using anti-IRAK-1 and anti-Myc antibodies to confirm expression of the constructs. Immunoreactivity was visualized by enhanced chemiluminescence.
FIGURE 6. IRAK-4 induces the degradation of Pellino proteins in a kinase-dependent but IRAK-1-independent manner. A, HEK293 cells were cotransfected with plasmid encoding Myc-tagged Pellino3L, Pellino2, Pellino1, or Pellino3L Y44A (0.4 μg) and the pcDNA3.1 empty vector (−) or a wild-type (WT) or kinase-dead (KD) IRAK-4 construct (0.6 μg). Cell lysates were generated the following day and subjected to PAGE and subsequently to Western immunoblotting using anti-Myc antibody. B, HEK293 cells were cotransfected using Lipofectamine 2000 with the designated Myc-tagged Pellino constructs or the pcDNA3.1 empty vector (−); 3 μg) and a FLAG-tagged or kinase-dead IRAK-4 expression construct (1 μg). Cell lysates were generated the following day and immunoprecipitated (IP) with anti-FLAG antibody. Immunoprecipitates were subjected to PAGE and subsequently to Western immunoblotting (WB) using anti-Myc and anti-IRAK-4 antibodies. Cell lysates were also analyzed by Western immunoblotting using anti-Myc antibody to confirm expression of the Pellino constructs. Immunoreactivity was visualized by enhanced chemiluminescence.

FIGURE 7. IRAK-2 interacts with Pellino proteins but fails to regulate their degradation. A, HEK293 cells were cotransfected with plasmid encoding Myc-tagged Pellino3L, Pellino3S, Pellino2, or Pellino1 (0.5 μg) or the pcDNA3.1 empty vector (EV) in the presence or absence of a construct expressing IRAK-2 (0.5 μg). Cell lysates were generated the following day and subjected to PAGE and subsequently to Western immunoblotting (WB) using anti-Myc antibody. B, HEK293 cells were cotransfected using Lipofectamine with the designated Myc-tagged Pellino constructs or the pcDNA3.1 empty vector (−); 2 μg) and a FLAG-tagged IRAK-2 expression construct (2 μg). Cell lysates were generated the following day and immunoprecipitated (IP) with anti-FLAG antibody. Immunoprecipitates were subjected to PAGE and subsequently to Western immunoblotting using anti-Myc and anti-FLAG antibodies. Cell lysates were also analyzed by Western immunoblotting using anti-Myc antibody to confirm expression of the constructs. Immunoreactivity was visualized by enhanced chemiluminescence.

This study provides the first direct evidence that Pellino proteins are E3 ligases. A previous report used an indirect approach to suggest that Pellino1 and Pellino2 (but not Pellino3) are E3 ligases that can polyubiquitinate IRAK-1 (28). However, we employed a direct in vitro approach to show that all members of the Pellino family are E3 ligases, with Pellino 3 displaying the greatest ligase activity. The inability of Pellino3 to promote polyubiquitination in the earlier study is surprising given that, in this study, we employed two independent approaches to unambiguously show that both forms of Pellino3 are E3 ligases. First, a direct in vitro ubiquitination assay demonstrated that recombinant and endogenous forms of Pellino3 have E3 activity. Second, overexpression of the Pellino3 spliced variants leads to polyubiquitination of IRAK-1. We have also shown for the first time that Pellino proteins, in conjunction with the E2 heterodimer UbcH13/Uev1a, catalyze Lys63-linked polyubiquitination. UbcH13/Uev1a was chosen as a lead E2 candidate
IRAKs Stimulate Pellino Polyubiquitination/Degradation

FIGURE 8. LPS induces the degradation of endogenous Pellino3 in PBMCs. Human PBMCs were stimulated with LPS (1 μg/ml; A) or TNF (100 ng/ml; B) for the indicated times. Cells were harvested in SDS sample buffer and subjected to PAGE and subsequently to Western immunoblotting using anti-Pellino3 and anti-β-actin antibodies. Immunoreactivity was visualized by enhanced chemiluminescence.

The inability of kinase-dead IRAK-1 and IRAK-4 to regulate polyubiquitination and degradation of Pellino proteins is not due to lack of interaction because we have shown that both kinase-dead forms interact with all members of the Pellino family. The importance of IRAK kinase activity for IRAK/Pellino interactions has been a source of some contention, with one report demonstrating that kinase activity is dispensable for interaction (25) and other reports showing it to be essential for IRAK members to display Pellino binding activity (26, 28). The basis of these contradictory reports is not understood. In this study, our data unambiguously favor a model in which the IRAK kinase activity is not required for manifesting IRAK/Pellino interactions but is essential for subsequent polyubiquitination and degradation of Pellino proteins. This is further supported by the ability of IRAK-2, an IRAK family member that lacks a functional kinase domain, to interact with the Pellino proteins but its failure to induce their degradation.

Because Pellino proteins have been implicated as mediators in TLR/IL-1R pathways that lead to activation of NF-κB and MAPKs, IRAK-induced degradation of Pellino proteins may represent a novel self-regulating mechanism for terminating these pathways. The demonstration of LPS-induced degradation of Pellino3 in PBMCs is consistent with such a model. Furthermore, it is interesting to note that kinase-dead IRAK-1 is more effective that its wild-type counterpart in activating NF-κB and JNK (19, 20). Whereas the molecular basis of this difference in efficacy is unknown, it is plausible that IRAK-1-induced degradation of Pellino proteins, coupled with the kinase dependence of this process, may contribute to wild-type IRAK-1 being less effective.

Since their discovery, no specific activity has been associated with members of the Pellino family, and thus, there has been a considerable gap in our understanding of the mechanism(s) by which Pellino proteins integrate into TLR/IL-1R pathways. This study has now assigned for the first time an E3 activity to IRAK-1 and IRAK-4.

The functional consequence of such polyubiquitination is likely to be the degradation of Pellino proteins because IRAK-1 induces the degradation of the Pellino family in a manner that is also dependent on IRAK-1 kinase activity. This capacity to induce Pellino degradation extends to IRAK-4, a known upstream regulator of IRAK-1. However, the effects of IRAK-4 on Pellino degradation are independent of IRAK-1, suggesting some functional redundancy between these two members of the Pellino family. The effects of IRAK-4 (like those of IRAK-1) are dependent on its kinase activity and an ability to interact

with the Pellino proteins, and our findings show that IRAK-mediated degradation of Pellino proteins is limited to IRAK members that possess a functional kinase domain. This study thus describes a novel role for the kinase activity of IRAK-1 and IRAK-4.

The interaction of IRAK-1 with the Pellino family members also leads to strong reciprocal polyubiquitination of the Pellino proteins. Although Pellino proteins were shown to be capable of catalyzing autoubiquitination in an in vitro setting, it is unlikely that IRAK-1-induced polyubiquitination of Pellino proteins is mediated by autoubiquitinating activity because mutation of the Pellino RING-like domain fails to abrogate IRAK-1-induced polyubiquitination of the Pellino family. Polyubiquitination of the Pellino family is dependent on the kinase activity of IRAK-1 and its association with the Pellino proteins, suggesting that IRAK-1-induced phosphorylation of Pellino proteins is a prerequisite to their polyubiquitination. The functional consequence of such polyubiquitination is likely to be the degradation of Pellino proteins by IRAK-1, a known upstream regulator of IRAK-1. However, the effects of IRAK-4 on Pellino degradation are independent of IRAK-1, suggesting some functional redundancy between these two members of the Pellino family. The effects of IRAK-4 (like those of IRAK-1) are dependent on its kinase activity and an ability to interact...
likely novel regulatory mechanism for tightly controlling these pathways.

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