Identification of Drosophila Yin and PEPT2 as Evolutionarily Conserved Phagosome-associated Muramyl Dipeptide Transporters

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The components of the innate immune system, including cellular defenses and inflammatory pathways, mount a rapid and tightly orchestrated response to pathogen invasion. Central to this is the activation of proinflammatory signals that are triggered by the simultaneous ligation of multiple pattern recognition receptors by ligands that are components of microbes and absent from the healthy host. The archetypal pattern recognition receptors are the Toll-like receptors (TLRs), transmembrane proteins found on the cell surface and in endosomal compartments. The NOD (nucleotide-binding oligomerization domain)-like receptors (NLRs), which reside in the cytosol and recognize bacteria, or their derivatives, that cross the plasma membrane and enter the cell. Despite the TLRs and NLRs surveying two discrete cellular compartments (the extracellular space and the cytosol), these receptors collaborate for optimal host response to many pathogens. However, how this collaboration is coordinated is poorly understood. Previous work has demonstrated that maximal activation of TLR and NOD2-dependent signaling in response to Gram-positive microbes is possible only after delivery into a phagosome and that this event is intimately associated with inflammatory cytokine induction. These observations reflect the multifunctional role of the phagosome during innate immune sensing of Gram-positive microbes. An emerging theme is that during maturation, the phagosome enzymatically modifies the cargo to liberate cryptic pattern recognition receptor ligands in a process we refer to as “pathogen processing.” These so-called post mortem PAMPS (PAMPs) include ligands for a wide variety of innate immune pattern recognition receptors including NOD2, the focus of this work.

NOD2 was identified less than a decade ago as a NF-κB activating protein. Subsequent genetic studies have revealed that mutations in NOD2 are linked to increased risk of development of Crohn’s disease. NOD2 is a multidomain protein containing two CARD domains, a central NOD domain and a C-terminal LRR domain that is thought to be the ligand-binding domain. Peptidoglycan-derived fragments, including the well characterized synthetic ligand muramyl dipeptide (MDP), are known ligands that activate NOD2 signaling pathways. Activation of NOD2 induces the production of multiple NF-κB-dependent cytokines, including IL-8 and IL-6, and antimicrobial peptides. Because of the association with inflammatory bowel disease, a major focus of work on NOD2 has been in understanding its role and regulation in epithelial cells. However, it is evident that NOD2 is important not only in epithelial

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2 The abbreviations used are: TLR, Toll-like receptor; NLR, NOD-like receptor; MDP, muramyl dipeptide; IL, interleukin; PBS, phosphate-buffered saline; IFN, interferon; YFP, yellow fluorescent protein; GFP, green fluorescent protein; TNF, tumor necrosis factor; CFP, cyan fluorescent protein; RT-PCR, real-time PCR.

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immunity but also in other cell types, including phagocytic antigen-presenting cells. Supporting this, NOD2 is expressed predominantly in myeloid cells (11), and macrophages derived from both Nod2-deficient mice and from Crohn’s disease patients carrying mutations in NOD2 are unable to activate NF-κB and mitogen-activated protein kinase (MAPK) pathways after MDP stimulation while retaining their capacity to respond to TLR ligands (16). Additionally, NOD2 is also involved in microbial sensing in a number of other systems, including a recently described role in sensing of viral pathogens (17), autophagy (18), and induction of adaptive immunity (19).

Intriguingly, despite being a cytosolic sensor, NOD2 participates not only in sensing cytoinvasive microbes but also in the sensing of extracellular pathogens. This fact raises the question of how the NOD2 ligands derived from extracellular microbes access intracellular compartments, and suggest that transport mechanisms must exist to translocate them across membranes to enter the cytosol surveilled by NOD2. As the ligands from extracellular pathogens appear to be generated by digestion and modification in phagolysosomes, we have focused on these organelles and set out to understand MDP transport in phagocytic cells. Specifically, we have addressed how phagosome-derived ligands access the cytosol. Drawing on data from Drosophila phagosomes, we identify an evolutionarily conserved role of SLC15A1 transporters, Drosophila Yin and PEPT2, as MDP transporters in fly and human phagocytes, respectively. Importantly, we show that these transporters associate with phagosome membranes and hence are likely to be of particular importance in delivery of ligands generated in this compartment to NOD2 recruited to the vicinity of these organelles.

**EXPERIMENTAL PROCEDURES**

**Mice and Cells**—C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Nod2−/− mice generated by K. Kobayashi. All mice were kept and handled under a protocol approved by the Subcommittee on Research Animal Care at Massachusetts General Hospital. Bone marrow-derived macrophages were generated by 7 days culture of bone marrow cells in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum and 20% L929 cell-conditioned medium with 1% fetal calf serum. The cells were stimulated with D. E. Golenbock (University of Massachusetts Medical School, Worcester, MA). GFP-tagged (pEG-FPC1-NOD2) NOD2 and nontagged NOD2 mammalian expression vectors were described previously (22). Full-length human SLC15A2 (PEPT2) from THP-1 cells and Drosophila Opt1/Yin from S2 cells were cloned into a Gateway entry vector (Invitrogen) by PCR using primers designed according to the manufacturer’s instructions. These entry clones were sequence verified and transferred into a Gateway mammalian expression vector (Invitrogen) by recombination. Human SLC15A1 was subcloned into Gateway vectors from a verified clone purchased from Open Biosystems (Huntsville, AL). Mammalian expression vector containing full-length human SLC15A3 coding sequence was purchased from Open Biosystems (clone ID, 5213632).

**Cell Stimulations and Treatments**—Macrophages in Dulbecco’s modified Eagle’s medium with 1% fetal calf serum were stimulated with heat-inactivated bacteria at a multiplicity of infection of 25:1, or MDP at the indicated concentrations, at 37 °C in 5% CO2 for 2 to 4 h for macrophages and 6 h for differentiated THP1 cells, after which culture supernatants were harvested. Cytokine secretion was measured by enzyme-linked immunosorbent assay (DuoSet Enzyme-linked Immunosorbent Assay Development System; R&D System, Minneapolis, MN) in accordance with the manufacturer’s protocol.

**Phagocytosis and Intracellular TNF-α and IL-6**—Macrophages in Dulbecco’s modified Eagle’s medium with 1% fetal calf serum were incubated with heat-inactivated bacteria labeled with tetramethylrhodamine (Molecular Probes, Eugene, OR), at a multiplicity of infection of 25:1 for 30 min on ice, allowing the synchronization of bacteria binding onto the cell. In all cases, before incubation with macrophages, bacterial clusters were disrupted by passing the bacteria through a 30-gauge needle. After 30 min on ice, the cells were further incubated for the indicated times at 37 °C in the presence of GolgiStop (BD Bioscience, San Diego, CA) to accumulate intracellular TNF-α. The cells were washed twice with ice-cold PBS containing 5 mM EDTA (PBS/EDTA), detached with scrappers, and fixed in 3% paraformaldehyde. The cells were permeabilized and stained with allophycocyanin-conjugated anti-mouse TNF-α or IL-6 antibody (BD Bioscience) diluted in PBS with 0.2% saponin. After washing, the cells were analyzed by flow cytometry performed on FACSCalibur (Becton Dickinson); analysis was performed with CellQuest Pro software (Becton Dickinson) to determine phagocytosis and intracellular TNF-α production at the single cell level.

**NF-κB Luciferase Reporter Assay**—Dual luciferase reporter assays for NF-κB activation were performed in HEK293T cells as described previously (23). Briefly, cells were transfected with the indicated expression constructs together with NF-κB luciferase (Firefly) and control luciferase (Renilla) reporter plasmids using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s protocol. Before assays, the cells were washed with PBS and cultured in fresh Dulbecco’s modified Eagle’s medium with 1% fetal calf serum. The cells were stimulated with heat-inactivated bacteria or bacterial ligands as described under “Cell Stimulations and Treatments.” Reporter gene activity was measured using the Dual-Glo luciferase assay system (Promega, Madison, WI) in accordance with the manufacturer’s protocol.
**Transporter Analysis**—Expression of SLC15 transporters was determined by semiquantitative RT-PCR using a Mastercycler ep realplex thermocycler (Eppendorf). Expression was normalized to glyceraldehyde-3-phosphate dehydrogenase and expressed as a percent of expression in kidney, which was used as a positive control for transporter expression. RT-PCR primers used to estimate relative expression of the different transporters are as follows: SLC15A1-F, 5'-GTTGCT-TCTGGTCTCTGTG-3'; SLC15A1-R, 5'-GCCCCTGAATGAAA-TATGG-3'; SLC15A2-F, 5'-GCCC-TGTCTTGAAAGCAATTTT-3'; SLC15A2-R, 5'-AGAGTCTCTG-GGCCTTGTT-3'; SLC15A3-F, 5'-GGCAA-GATCTTCACCAGCAC-3'; SLC15A3-R, 5'-GGCAA-GATCTTCACCAGCAC-3'; SLC15A4-F, 5'-AGCGATCCTGT-CGTTAGGTG-3'; SLC15A4-R, 5'-AGGAGGCTTGAGATGAGAAAA-3'.

**Microscopy**—HEK293T cells were transiently transfected with pDEST53-PEPT2 to allow expression of a GFP-PEPT2 fusion protein. The cells were than incubated for 2 h with red fluorescent 1 μm diameter latex beads (Estapore, Paris, France) to allow engulfment. Immunofluorescence studies were performed on cells fixed in 4% paraformaldehyde (Sigma-Aldrich) or methanol (for anti-PEPT2 and anti-NOD2 staining) and imaged with a confocal microscope (Bio-Rad/Zeiss Radiance 2000 or Nikon TiE with a Perkin Elmer spinning disc). Nonconfocal images were acquired using a Nikon inverted microscope equipped with a CoolSnap camera. Post-acquisition image analysis was performed with Adobe Photoshop, Openlab, or Velocity software (Improvision).

**Statistical Analysis**—All experiments were performed in triplicates and repeated at least three times.
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independently. Statistical significance was determined by using a two-tailed unpaired Student’s t test.

RESULTS

Response to S. aureus Is Mediated by the Collaboration between TLR2/6 and NOD2 from the Phagosome—NF-κB-driven responses to S. aureus are mediated primarily by membrane-bound TLRs that respond to immunostimulatory components of the Gram-positive bacterial cell wall that include lipoteichoic acid, peptidoglycan, and lipopeptides (24). However, a second family of pattern recognition receptors, the cytosolic NLRs, has also been suggested to participate in response to these primarily extracellular microbes. Specifically, it is believed that NOD2 senses a peptidoglycan derivative, similar to MDP that is released from the bacterial cell wall (25). To further define the role of NOD2 in the response to S. aureus, we isolated macrophages from Nod2−/− mice and measured TNF-α and IL-6. Resting Nod2−/− macrophages showed no defect in response to S. aureus, but when primed with IFNγ, showed impaired response compared with wild-type controls (Fig. 1a). To determine the relative contributions of the TLR and NOD2 pathways to S. aureus sensing, we next used HEK293T transfected with TLR2/6 and/or NOD2 and measured NF-κB activation. Transfection of NOD2 alone was unable to render HEK293T cells responsive to S. aureus (Fig. 1b). However, when coexpressed with TLR2/6, NOD2 enhanced NFκB activation, indicating that although NOD2 is insufficient for full activation, it synergized with TLR2/6 to increase cytokine response. Thus, the collaboration of TLRs with NOD2 is necessary for optimal response to S. aureus in both the mouse and human systems.

NOD2 is normally distributed in the cytosol and weakly associated with the plasma membrane in resting cells (22, 26). However, for signaling to occur, NOD2 must be recruited and tethered to membranes along with its signaling adaptor, RIP2 (26). Our previous work (21, 23) and the work of others (6, 7) have shown that TLR2 and its co-receptors CD36 and MBL are internalized after uptake of particulate ligands (6, 7, 21, 23). We therefore hypothesized that, like other components of the S. aureus sensing machinery, NOD2 may also be recruited to phagosome membranes. Consistent with this possibility, ectopically expressed GFP-NOD2 concentrated in close proximity to phagosomes generated in HEK293T cells (Fig. 1c). Similarly, endogenous NOD2 was also found associated with phagosomes in THP1 human macrophages (Fig. 1d).

As NOD2 is recruited to phagosomes, which also generate the NOD2 ligand (8), we next tested whether these organelles were the origin of NOD2 activation after internalization of S. aureus. Our previous work (21) has shown that TNF-α and IL-6 response to S. aureus is triggered primarily from phagosomes, and, hence, response to these bacteria can be used as a surrogate for phagosome-dependent signaling. Therefore, using a single cell fluorescence-activated cell sort-e based assay in which cytokine production and phagocytosis can be monitored simultaneously (21), we tested whether NOD2-dependent TNF-α and IL-6 production in response to internalized S. aureus was impaired in macrophages from Nod2−/− mice (Fig. 1e). Phagosome-triggered cytokine production, particularly IL-6, was markedly impaired in Nod2−/− macrophages. Together with our data that NOD2 is recruited to phagosomes, these observations support the hypothesis that NOD2 activation occurs primarily from these organelles (27). However, despite recruitment to phagosomes, NOD2 remains on the cytosolic aspect of the membrane. This observation raises the question of how cytosolic NOD2 participates in cytokine signaling, as it is an extracellular pathogen that does not normally access the cytosol. We therefore set out to establish how ligands derived from extracellular bacteria such as S. aureus access NOD2, a cytosolic sensor in human cells.

Identification of Drosophila Yin as a Phagosome-associated MDP Transporter—For NOD2 to be activated, the bacteria-derived ligands (such as MDP and other glycopeptides) must first be transported out of the phagosome into the cytosol. To identify potential phagosome-associated transporters, we took advantage of a database of phagosome-associated proteins derived from proteomic analysis of Drosophila phagosomes (23). Interrogation of that database identified a number of candidate transporters (Fig. 2a). One particular protein, Yin (found in the 30 min and 2 h Drosophila phagosome proteome), was identified as a particularly promising candidate. Yin is related to a family of SLC15A transporters that are best known for their role in nutrient acquisition in the gastrointestinal tract and are considered as a promising drug delivery target in colonic and kidney epithelial cells. One member of this family, PEPT1, has

| Uniprot-ID | Flybase ID | Name | Predicted molecular function |
|-----------|------------|------|-------------------------------|
| P91679    | CG2913     | Yin  | Transporter - AA + SLC15A type |
| Q9V627    | CG12317    | Jil21| Transporter - AA             |
| Q9K418    | CG3297     | Hiplace| Transporter - AA             |
| Q0Y489    | CG7282     | C9DH heavy chain | Transporter - AA + ions |
| Q5V8M4    | CG13384    | SGC13384 | Transporter - AA + proline |
| Q5V1M1    | CG14709    | C9G14709 | Transporter - ABC |
| Q0Y478    | CG9281     | C9G281 | Transporter - ABC             |
| Q5V8R5    | CG6214     | C9G6214 | Transporter - ABC (MDR)       |
| Q5V6E5    | CG5789     | C9G5789 | Possible ABC transporter |
| Q86S8F5   | CG3164     | C9G3164 | Possible ABC transporter        |
| Q5T0R4    | CG7627     | C9G7627 | Possible ABC transporter       |

FIGURE 2. Identification of Yin, a phagosome-associated MDP Drosophila transporter. a, the table summarizes all of the proteins identified from our previous proteomic analysis of the Drosophila phagosome (23) that are transporter candidates. Names and predicted molecular function were extracted from the Flybase database. b, NOD2 and an NF-κB reporter construct were expressed in HEK293T cells with and without Drosophila Yin, a potential MDP transporter. Sensitivity to MDP was determined by luciferase activity. Graphs are representative of at least three independent experiments. Mean ± S.D. are represented. **, p ≤ 0.01 relative to NOD2 + MDP. RLU, relative light units.
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SLC15 family members in kidneys, THP1 (human monocyte/macrophage cell line), peripheral blood monocytes, Caco2 (colonic epithelial cell line), and HEK293T (human kidney epithelial cell line) was determined by 40 cycle RT-PCR (a) and quantitative RT-PCR (c). PEPT2, but not PEPT1, is expressed by human phagocytes, which also express SLC15A3 and SLC15A4.

Yin and expressed it along with NOD2 and a NF-κB reporter in mammalian HEK293T cells. Similar to Yin, transfection of PEPT2 with NOD2 greatly increased the sensitivity of HEK293T NF-κB reporter cells to exogenous MDP (Fig. 4a). Importantly, PEPT2 was as efficient at increasing the sensitivity of HEK cells to MDP as PEPT1, a bona fide MDP transporter expressed by colonic epithelial cells (28). In contrast, the more distantly related transporter, SLC15A3, weakly modified NOD2 activation in the same system (Fig. 4b). Thus, similar to PEPT1 and Yin, PEPT2 is also a MDP transporter.

MDP Transport by PEPT2 and NOD2 Activation Is pH-sensitive—SLC15A transporters symport di- and triptides with H⁺ using energy provided by the proton gradient. In vivo, the proton gradients across epithelial cells are maintained by the existence of tight junctions between the cells in monolayer, along with its high expression in human phagocytes, led us to reason that PEPT2 might be a candidate phagosome-associated MDP transporter in human cells.

To test whether PEPT2 could transport MDP, we next expressed PEPT2 along with NOD2 and a NF-κB reporter in mammalian HEK293T cells. Similar to Yin, co-transfection of PEPT2 with NOD2 greatly increased the sensitivity of HEK293T NF-κB reporter cells to exogenous MDP (Fig. 4a). Importantly, PEPT2 was as efficient at increasing the sensitivity of HEK cells to MDP as PEPT1, a bona fide MDP transporter expressed by colonic epithelial cells (28). In contrast, the more distantly related transporter, SLC15A3, weakly modified NOD2 activation in the same system (Fig. 4b). Thus, similar to PEPT1 and Yin, PEPT2 is also a MDP transporter.

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enriched on phagosome membranes (Fig. 6a). Using an anti-PEPT2 antibody, we next determined the localization of endogenous PEPT2 in the human THP1 monocyte-derived cell line during phagocytosis (Fig. 6b). Similar to the localization of GFP-PEPT2 in HEK293T cells, PEPT2 was found associated with the plasma membrane in resting macrophages and also enriched in phagocytic cups and phagosomes after particle internalization. Thus, the high levels of PEPT2 expression in human macrophages, the ability of PEPT2 to increase NOD2 response, and its association with phagosome membranes suggest this as a likely phagosome-associated MDP transporter in human phagocytes.

DISCUSSION

NOD2 is a cytosolic sensor and thus does not readily access extracellular bacteria. For this reason, a number of studies have focused on identifying the mechanism by which NOD2 ligands access the cytosol. Because of the association of NOD2 mutations and gastrointestinal inflammation, these studies have been performed primarily in epithelial cells of the colon or kidney. The consensus is that both NOD1 and NOD2 ligands enter these epithelial cells by endocytosis (30, 32). Additionally, these studies have converged on a family of SLC15A transporters as potential mechanisms to transport NOD1 and NOD2 ligands across membranes; PEPT1 has been shown to transport MDP across the plasma membrane of colonic epithelial cells to activate cytosolic NOD2, whereas SLC15A4 has been implicated in transport of NOD1 ligands (28–30). However, studies of NLR ligand transport using nonepithelial cells have been limited. One study addressing this in macrophages has shown that NOD2-dependent signaling is unimpaired in PEPT1 deficient macrophages, suggesting that this is not an important transporter in these cells (31). Pannexin-1, a P2X7-interacting hemichannel, has been shown to be an NLR ligand transporter in macrophages and required for MDP to trigger NLRP3-dependent IL-1β secretion (32, 33). However, pannexin-1 does not transport MDP for activation of NFkB by NOD2. Thus,
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although it has been shown that purified MDP is endocytosed by macrophages or that NOD2 ligands can be derived from bacteria in the phagolysosome (8), the mechanisms of membrane translocation of MDP in professional phagocytes remains to be fully defined. Here, we identify Yin and PEPT2 as evolutionarily conserved transporters of MDP. Importantly, these transporters are highly expressed by professional phagocytes, where they are recruited to phagosomes. These observations lead us to suggest that Yin and PEPT2 may be of particular importance in transporting ligands derived from internalized bacteria to activate the cytosolic pattern recognition receptor in flies (34). It will be of interest to see whether Yin plays a role in transporting tracheal cytotoxin to activate peptidoglycan recognition protein-LE in a manner analogous to the role we propose for PEPT2 in NOD2 activation in human cells. Additionally, our work studying these transporters in mouse macrophages is also of interest. Despite being sensitive to MDP, we were unable to detect significant levels of either Pept1 or Pept2 in both J774 and elicited peritoneal macrophages (data not shown). These data indicate that mouse phagocytes may use different mechanisms of MDP transport from humans. Of note, these cells did express Slc15a3 and a4, and it is possible that these or other transporters are also able to function as phagosome-associated MDP transporters and partially compensate for the lack of Pept2 in these cells. Intriguingly, when considered with the Drosophila data, these observations suggest that mice have selectively lost expression of this transporter in phagocytes. It is tempting to speculate that the loss of Pept2 might decrease the efficiency of MDP translocation in mouse macrophages and contribute to the relative resistance of these animals to septic shock (35), thus providing them with a survival advantage during certain infectious challenges.

Consistent with redundancy of these transport mechanisms, our gain-of-function experiments clearly show a role of PEPT2 in NOD2 ligand delivery in human cells, whereas our loss of function RNA interference experiments, in which we have attempted to silence PEPT2, have been inconclusive (data not shown). PEPT2 is not the only member of the SLC15 transporter family expressed by phagocytes, and the observed redundancy is most likely due to compensation by other SLC15A transporters. We find that although PEPT1 is expressed only at very low levels it may nonetheless be sufficient to compensate and transport MDP in the absence of PEPT2. Macrophages also express SLC15A3 and A4. However, ectopic expression of SLC15A3 did not increase sensitivity of HEK293T cells to the NOD2 ligand in our system. Although we have not formally tested the role of SLC15A4 in MDP transport, work by Lee et al. (30) has shown that SLC15A4 is a transporter for NOD1 ligands such as M-Tri-DAP but does not have a clear role in transport of MDP. Therefore, it is not clear which of the SLC15A transporters may be compensating for PEPT2 in macrophages. SLC15A transporters have broad substrate specificity for di- and tripeptides and can transport a variety of molecules with varying efficiency. This pleiotropism raises the question of whether PEPT2 functions only to transport MDP or might have additional roles in transport of other innate immune agonists. Work by Swaan et al. (36) compared the relative efficiency of PEPT2 in transport of γ-iE-DAP and MDP and showed that γ-iE-DAP was a better substrate for ectopically expressed PEPT2 in CHO-K1 cells than MDP. Because of our focus on NOD2 we have not tested the role of PEPT2 in transport of γ-iE-DAP but, considering these observations, it is likely that this transporter may also transports NOD1 ligands from the phagosome.

In summary, we identify here Drosophila Yin and human PEPT2 as an evolutionarily conserved mechanism for the transport of MDP into the cytosol of professional phagocytes, where it is recognized by NOD2. PEPT2 transport is pH-dependent.
and hence is likely to rely on proton gradients generated either in the endolysosomal compartment or during maturation of phagosomes. Importantly, we show that along with NOD2, PEPT2 is enriched around phagosomes. Our results emphasize the importance of considering not only the affinity of the transporters for their substrates but also the patterns of gene expression and the subcellular localization of these proteins when trying to understand how SLC15A transporters function to translocate NOD ligands into cells. The delivery of NOD ligands appears to be mediated by dedicated transporters such as PEPT2, which, therefore, represent potential therapeutic targets to decrease the detrimental effects of MDP and other agonistic fragments derived from bacteria during septic shock. These data indicate that the phagosome compartmentalizes BIAL sensing of recruited to the vicinity of the organelle. Moreover, these data show that most of the machinery required for optimal microbial sensing of S. aureus is recruited to the vicinity of this highly hydrolytic organelle, greatly increasing the efficiency of signaling after bacterial internalization.

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