

# The Innate Immune Protein Nod2 Binds Directly to MDP, a Bacterial Cell Wall Fragment

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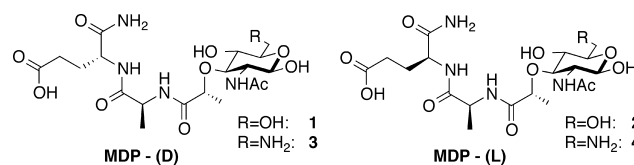
## S Supporting Information

**ABSTRACT:** Mammalian Nod2 is an intracellular protein that is implicated in the innate immune response to the bacterial cell wall and is associated with the development of Crohn's disease, Blau syndrome, and gastrointestinal cancers. Nod2 is required for an immune response to muramyl dipeptide (MDP), an immunostimulatory fragment of bacterial cell wall, but it is not known whether MDP binds directly to Nod2. We report the expression and purification of human Nod2 from insect cells. Using novel MDP self-assembled monolayers (SAMs), we provide the first biochemical evidence for a direct, high-affinity interaction between Nod2 and MDP.

The innate immune system is the body's first line of defense against invading pathogens.<sup>1,2</sup> This ancient system has evolved to exist in a symbiotic relationship with commensal bacteria and at the same time to recognize and destroy virulent bacteria.<sup>1–3</sup> Chronic inflammatory diseases such as asthma, rheumatoid arthritis, and Crohn's disease are thought to arise from an inappropriate innate immune response to bacteria.<sup>4–9</sup> Chronic inflammation has also been shown to lead to a variety of types of cancers, including gastric, colon, and lung cancer.<sup>10</sup>

Mammalian Nod2 is an intracellular protein that is involved in the signaling response to bacterial cell wall fragments.<sup>11,12</sup> Mutations in Nod2 correlate with the development of Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract.<sup>13–15</sup> To generate the proper immunological response, the Nod2 signaling pathway must recognize bacteria. The biochemical mechanism by which Nod2 detects bacteria is not known, but it has been proposed that Nod2 senses bacterial cell wall fragments directly. Nod2 could sense bacterial cell wall fragments through at least three mechanisms: (1) a direct interaction, (2) a mediated interaction, or (3) a signaling relay. The mechanism of activation remains unresolved because until now the proper tools to probe the mechanism have been unavailable.

Nod2 is essential for the cellular response to a small fragment of bacterial cell wall, muramyl dipeptide (MDP), consisting of one carbohydrate and two amino acids. The biologically relevant isomer is MDP-(D) (1) (Figure 1).<sup>12,16</sup> MDP is found in both Gram-negative and Gram-positive bacteria. Cellular and in vivo assays have shown that when mammalian cells expressing Nod2 are treated with MDP, an inflammatory



**Figure 1.** Muramyl dipeptides. MDP-(D) is the biologically relevant isomer; MDP-(L) is a synthetic diastereomer of the compound found in nature.

response is activated via the NF- $\kappa$ B and MAP kinase pathways.<sup>17–20</sup> Moreover, the response is not observed if MDP-(L) (2), a diastereomer of 1 (Figure 1), is used in the cellular assays. For this reason, in the literature MDP is often called the “ligand” for Nod2 signaling.<sup>21</sup> However, there are no biochemical or biophysical data that demonstrate an interaction between the two molecules.<sup>22</sup> The purpose of our investigation was to determine whether Nod2 and MDP interact in vitro. We developed an expression system and a biochemical assay using synthetic probes to investigate this question.

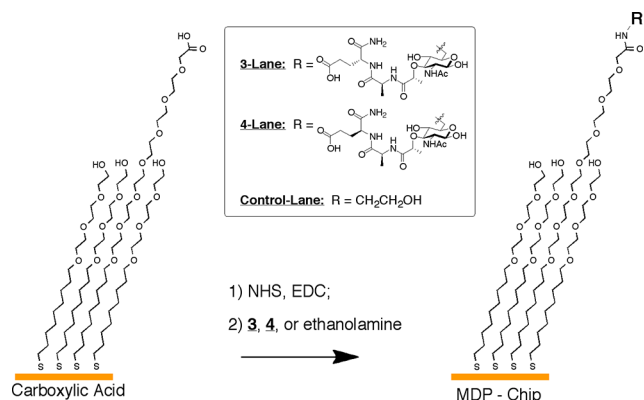
Human Nod2 is a large protein (1040 residues, 110 kD) with multiple domains: two N-terminal caspase recruitment domains (CARDs), a central nucleotide oligomerization domain (NOD), and a C-terminal leucine-rich repeat (LRR) domain.<sup>16</sup> To determine whether Nod2 interacts directly with MDP, we first expressed a Flag-tagged version of Nod2 using baculovirus-infected Sf21 cells [Supporting Information (SI) Figure 1a] with a yield of 1 mg/L. Circular dichroism spectroscopy and limited proteolysis experiments were consistent with Nod2 being a folded protein (SI Figure 1b,c).

With purified Nod2 in hand, we developed a surface plasmon resonance (SPR) assay to assess binding to MDP. Initial attempts to develop an SPR assay with biotinylated MDP<sup>23</sup> failed, as we observed significant nonspecific binding of Nod2 to the streptavidin/biotin chip lacking MDP (SI Figure 2). To develop the SPR assay, we coupled 6-amino-MDP (3 and 4; Figure 1) directly to the chip without the use of biotin. 3 and 4 are synthetic intermediates of the biotinylated MDPs that have been shown to activate Nod2 in the appropriate manner.<sup>23</sup> Using methodology developed by Whitesides and co-workers, we prepared carboxy-terminated alkanethiol self-assembled monolayers (SAMs) and then used on-chip *N*-hydroxysuccini-

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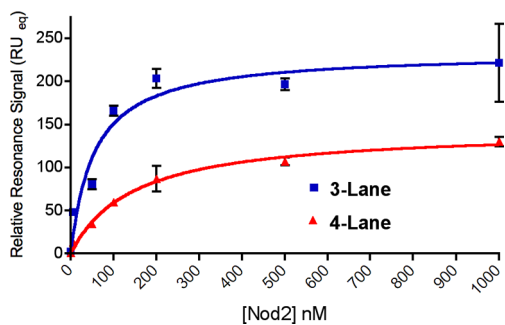
amide (NHS)/1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) activation of the carboxylic acid (Figure 2)<sup>24</sup> to couple the 6-amino-MDPs to the chip surface.



**Figure 2.** Synthesis of the MDP chip. A mixed SAM consisting of 1 mol % hexa(ethylene glycol)carboxylic acid [(EG)<sub>6</sub>CO<sub>2</sub>H]-terminated thiol in tri(ethylene glycol) [(EG)<sub>3</sub>OH]-terminated thiol was prepared. The carboxylic acid groups were then activated with NHS and EDC to form NHS esters. Displacement of NHS esters with the amino groups of MDPs formed amide bonds. Excess NHS esters were deactivated with ethanolamine.

A typical SPR assay uses four sensor lanes on a single chip.<sup>25</sup> In the assay, we included two controls: (1) the isoglutamine diastereomer of MDP (4; Figure 1), which does not activate the Nod2 pathway, and (2) an ethanolamine-capped monolayer (Figure 2). A typical assay setup involved flowing Nod2 over each lane of the sensor chip and observing changes in resonance units (RU). The assay was robust and allowed the screening of a wide variety of conditions. There was lower background binding of Nod2 to the synthetic chip relative to the biotin chip (SI Figures 2 and 3).

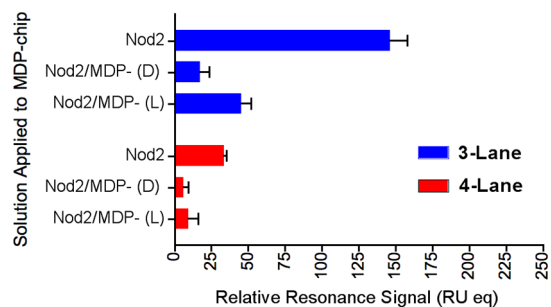
Nod2 bound to MDP with high affinity (Figure 3). The biologically active MDP, 3-Lane, bound to Nod2 with  $K_D = 51$



**Figure 3.** Nod2 binds to MDPs 3 and 4 with high affinities. Nod2 (pH 6.0) was applied to the MDP chip at varying concentrations at a rate of 3 mL min<sup>-1</sup>. After 10 min, the resonance signal was recorded and then plotted after subtraction of the ethanolamine background. The data were fitted to a standard one-site binding model by nonlinear regression analysis using Prism 4 (GraphPad).

$\pm 18$  nM. Surprisingly, we found that Nod2 was able to bind to both isomers of MDP (Figure 3), as Nod2 bound to 4-Lane with  $K_D = 150 \pm 24$  nM, which is only slightly higher than the  $K_D$  observed for 3-Lane. Thus, the isoglutamine stereochemistry is not a key recognition determinant. To demonstrate that binding of Nod2 to the MDP chip was

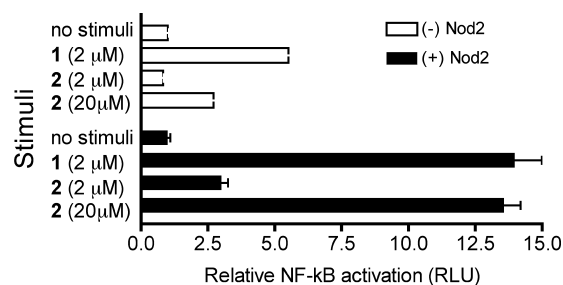
indeed specific, a competition study was performed. When Nod2 was pretreated with either 3 or 4, diminished binding to the chip was observed (Figure 4). Encouragingly, the



**Figure 4.** Binding of Nod2 to the MDP chip is specific. Nod2 [pH 5.5, 0.5  $\mu$ M, in either the presence or the absence of free MDP (1  $\mu$ M)] was applied to the MDP chip. The relative resonance signal was recorded after 10 min.

competition data showed the same trend as the on-chip data, namely, that the free D isomer of MDP is a better competitor than the free L isomer. These data suggest that Nod2 senses bacterial cell wall fragments by binding directly to them.

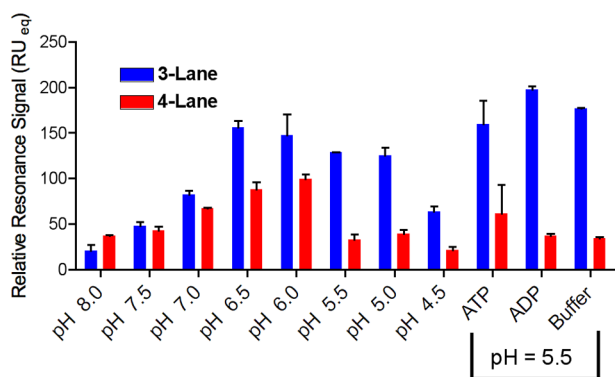
At low concentrations, the MDP isomers 2 and 4 do not activate the NF- $\kappa$ B response via Nod2 in cellular assays.<sup>18,23</sup> However, using the established cellular NF- $\kappa$ B luciferase reporter assay and transfected Nod2, we showed that MDP-(L) is able to activate the pathway at higher concentrations (Figure 5). The NF- $\kappa$ B activation observed in the absence of



**Figure 5.** MDP-(L) (2) activates the Nod2/NF- $\kappa$ B pathway at higher concentrations. Hek293T cells were transfected with ( $\pm$ )-Nod DNA (0.1 ng), NF- $\kappa$ B reporter, and a Renilla control. The cells were treated with stimuli for 12 h, harvested, and tested for luciferase activity.

transfected Nod2 DNA is the result of low levels of endogenous Nod2 in Hek293T cells.<sup>26</sup> The cellular assay results demonstrate that both isomers of MDP are able to activate the Nod2/NF- $\kappa$ B pathway, which is consistent with the binding data showing that Nod2 can bind one isomer better than the other. Cellular potencies often do not exactly match their *in vitro*  $K_D$  values.<sup>27,28</sup> We found that the cellular activation parallels the *in vitro* binding but at slightly lower potency.

The *in vitro* interaction between Nod2 and MDP is pH-dependent (Figure 6), with the pH range from 5.0 to 6.5 giving maximal binding. The data suggest that *in vivo* binding could occur in an acidic cellular compartment, a model supported by cellular assays showing pH-dependent internalization of MDP.<sup>29,30</sup> Girardin and co-workers showed that the internalization of MDP is optimal in the pH range from 5.5 to 6.5, which corresponds with the MDP/Nod2 binding data.



**Figure 6.** Nod2 and MDPs interact under a variety of conditions. The pH of Nod2 was adjusted before application to the MDP chip. Nod2 was preincubated with 10  $\mu$ M ATP or ADP before application to the MDP chip. The relative resonance signal was recorded after 10 min.

Nod2 is predicted to have ATP binding capabilities, as it has Walker A and Walker B regions.<sup>31</sup> The ATP binding capacities of the protein have been suggested to be important for oligomerization, protein–protein interactions, and subsequent activation.<sup>32</sup> To test whether nucleotide binding is necessary for Nod2 to bind to MDP, we measured Nod2 binding to MDP with and without ATP/ADP. Nod2 binds to MDP with no appreciable change in the presence and absence of 10  $\mu$ M ATP/ADP (Figure 6), suggesting that ATP/ADP is not necessary for binding of Nod2 to MDP.

Prior to our investigation, the mechanism of Nod2 activation of NF- $\kappa$ B by treatment with MDP was unclear. We have employed a biochemical approach to demonstrate that Nod2 binds directly to bacterial cell wall fragments. Recombinant Nod2 and the synthetic MDP tools allowed for the development of in vitro assay to detect binding. The assay that we have developed will be a valuable asset in screening for inhibitors/activators of the Nod2 signaling pathway and determining whether Nod2 is able to differentiate commensal versus pathogenic bacteria. In addition, the assay will be useful in determining whether the Nod2 Crohn's mutants are capable of binding to MDP. This is the first biochemical evidence to show an interaction between the two molecules, and it establishes that MDP is a high-affinity ligand for Nod2.

## ■ ASSOCIATED CONTENT

### Supporting Information

Nod2 expression and purification conditions, SPR assay, and NF- $\kappa$ B assay conditions. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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### Notes

The authors declare no competing financial interest.

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