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Accessibility
The innate immune protein Nod2 binds directly to MDP, a bacterial cell wall fragment

Catherine Leimkühler Grimes†§, Lushanti De Zoysa Ariyananda§, James E. Melnyk§, and Erin K. O’Shea†

†Department of Chemistry and Biochemistry, University of Delaware, Newark, DE 19716. ‡ Howard Hughes Medical Institute, Harvard Faculty of Arts and Sciences Center for Systems Biology, Departments of Molecular and Cellular Biology and of Chemistry and Chemical Biology, Harvard University, Cambridge, MA 02138.

Supporting Information Placeholder

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 if 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.

Figure 1: Muramyl Dipeptides: MDP-(D) is the biologically relevant isomer; MDP-(L) is a synthetic diastereomer of the compound found in Nature.

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 10. To determine if Nod2 interacts directly with MDP we first expressed a Flag-tagged version of Nod2 using baculovirus-infected Sf21 cells (Figure S1a in the Supporting Information) with a yield of 1 mg/L. CD spectroscopy and limited proteolysis experiments are consistent with Nod2 being a folded protein (Figure S1b and S1c in the Supporting Information).

With purified Nod2 in hand, a Surface Plasmon Resonance (SPR) assay was developed to assess binding to MDP. Initial attempts to develop a SPR assay with biotinylated-MDP† failed, as we observed significant non-specific binding of Nod2 to the streptavidin/biotin chip lacking MDP (Figure S2). In order to develop a SPR assay, we coupled 6-amino MDP (3 & 4, Figure 1) directly to the chip without the use of biotin. 3 & 4 are synthetic intermediates of the biotinylated-MDPs and have been shown to activate Nod2 in the appropriate manner 23. Using methodology developed by Whitesides and co-workers, we prepared carboxy-terminated alkane thiol self assembled monolayers (SAMs) and then used on-chip NHS/EDC activation of the carboxylic acid (Figure 2) 24 to couple 6-amino-MDPS to the chip surface.
A typical SPR assay uses four sensor lanes on a single chip. In the assay, we included two controls: (1) the iso-glutamine diastereomer of MDP (4, Figure 1) that 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 as compared to the biotin chip (Figure S2 and S3).

At low concentrations the MDP isomers 2 and 4 do not activate the NF-kB response via Nod2 in cellular assays. However, we show that MDP-4(L) is able to activate the pathway at higher concentrations using the established cellular NF-kB luciferase reporter assay and transfected Nod2 (Figure 5). The NF-kB activation observed in the absence of transfected Nod2 DNA is the result of low levels of endogenous Nod2 in Hek293T cells. The cellular assay results demonstrate that both isomers of MDP are able to activate the Nod2/NF-kB 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<sub>D</sub> values.

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Figure 4: Binding of Nod2 to the MDP-chip is specific. Nod2 (pH 5.5, 0.5 μM, either in the presence or absence of free MDP (1 μM)) was applied to the MDP-Chip. The relative resonance signal was recorded after ten minutes.

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oligomerization, protein-protein interactions and subsequent activation\textsuperscript{12}. To test if the nucleotide binding was necessary for Nod2 to bind to MDP, we measured Nod2 binding to MDP ± ATP/ADP. Nod2 binds with no appreciable change to MDP in the presence and absence of 10 μM ATP/ADP (Figure 6), suggesting that ATP/ADP is not necessary for Nod2 to bind to MDP.

![Figure 6: Nod2/MDPs interact under a variety of conditions. The pH of Nod2 was adjusted before application to the MDP-Chip. Nod2 was pre-incubated with 10 μM ATP or ADP before application to the MDP-Chip. The relative resonance signal was recorded after ten minutes.](image)

Prior to our investigation, the mechanism of Nod2 activation of NF-κB by treatment with MDP was unclear. We have taken 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 \textit{in vitro} assay to detect binding. The assay that we have developed will be a valuable asset in screening for inhibitory/activators of the Nod2 signaling pathway and determining if Nod2 is able to differentiate commensal versus pathogenic bacteria. In addition, the assay will be useful in determining 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 establishes that MDP is a high-affinity ligand for Nod2.

**ASSOCIATED CONTENT**

**Supporting Information.** Nod2 expression and purification conditions, SPR assay, and NF-κB assay conditions: this material is available free of charge via the Internet at http://pubs.acs.org.

**AUTHOR INFORMATION**

**Corresponding Author**
* egirmes@udel.edu

**Author Contributions**
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

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**ABBREVIATIONS**

MDP, muramyl dipeptide; SPR, surface Plasmon resonance; SAMs, self assembled monolayers (SAMs)

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