Molecular basis for peptidoglycan recognition by a bactericidal lectin

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RegIII proteins are secreted C-type lectins that kill Gram-positive bacteria and play a vital role in antimicrobial protection of the mammalian gut. RegIII proteins bind their bacterial targets via interactions with cell wall peptidoglycan but lack the canonical sequences that support calcium-dependent carbohydrate binding in other C-type lectins. Here, we use NMR spectroscopy to determine the molecular basis for peptidoglycan recognition by HIP/PAP, a human RegIII lectin. We show that HIP/PAP recognizes the peptidoglycan carbohydrate backbone in a calcium-independent manner via a conserved “EPN” motif that is critical for bacterial killing. While EPN sequences govern calcium-dependent carbohydrate recognition in other C-type lectins, the unusual location and calcium-independent functionality of the HIP/PAP EPN motif suggest that this sequence is a versatile functional module that can support both calcium-dependent and calcium-independent carbohydrate binding. Further, we show HIP/PAP binding affinity for carbohydrate ligands depends on carbohydrate chain length, supporting a binding model in which HIP/PAP molecules “bind and jump” along the extended polysaccharide chains of peptidoglycan, reducing dissociation rates and increasing binding affinity. We propose that dynamic recognition of highly clustered carbohydrate epitopes in native peptidoglycan is an essential mechanism governing high-affinity interactions between HIP/PAP and the bacterial cell wall.

T he intestinal epithelium is the major interface with the indigenous microbiota and a primary portal of entry for bacterial pathogens. To defend against continual microbial challenges, intestinal epithelial cells produce a diverse repertoire of secreted protein antibiotics that protect against enteric infections and limit opportunistic invasion by symbiotic bacteria. We previously identified lectins as a distinct class of secreted antibacterial proteins made in the mammalian intestinal epithelium (1). Members of the C-type lectin family share a common protein fold, the C-type lectin-like domain (CTLD), that is defined by a set of conserved sequences (2). Many C-type lectin family members bind carbohydrate ligands in a calcium (Ca\(^{2+}\))-dependent manner through a distinct set of conserved residues that govern both Ca\(^{2+}\)-dependence and carbohydrate ligand specificity (2). The Reg (regenerating) family constitutes a unique group of mammalian CTLD-containing proteins that consist solely of a ~16-kD CTLD and N-terminal secretion signal. Despite having a canonical C-type lectin fold, Reg proteins lack conserved sequences that support Ca\(^{2+}\)-dependent carbohydrate binding in other C-type lectins (2). Several RegIII proteins are highly expressed in the small intestine, including mouse RegIIIγ and human HIP/PAP (hepatointestinal pancreatic/pancreatitis associated protein) (1, 3). We have shown that RegIIIγ and HIP/PAP are directly bactericidal for Gram-positive bacteria at low micromolar concentrations (1, 4), revealing a unique biological function for mammalian lectins.
of the carbohydrate (5, 6). Sugar binding and specificity is dictated by positioning of the hydrogen-bond donors and acceptors in the residues flanking the conserved proline, and is determined by the orientations of the sugar 3- and 4-OH groups. Selective binding is conferred by the Loop 2 tripeptide motif with the orientations of the sugar 3- and 4-OH groups, and the OPD motif binding GalNAc or galactose (which have axial 4-OH groups) (5) (Fig. 1). While RegIIIy and HIP/PAP selectively recognize GlcNAc or mannose polysaccharides, they do so in a Ca\(^{2+}\)-independent manner (1, 8). Furthermore, both proteins lack the Loop 2 EPN and the \(\beta\) ND motifs (Fig. 1B), suggesting a distinctive mode of carbohydrate recognition.

**Peptidoglycan Induces Conformational Changes in a Loop 1 EPN Motif.** We used solution nuclear magnetic resonance (NMR) spectroscopy as an unbiased approach for identifying HIP/PAP residues involved in peptidoglycan binding. Chemical shift perturbations of backbone amide resonances, observed in \(^{15}N/H\) HSQC spectra, are sensitive indicators of changes in chemical environment at specific amino acid residues. We titrated solubilized peptidoglycan (sPGN) into \(^{15}N\)-labeled HIP/PAP and monitored chemical shift and linewidth changes in \(^{15}N/H\) HSQC spectra. As we had previously assigned the backbone chemical shifts of 98% of the HIP/PAP \(^{15}N/H\) resonances (4), we were able to map these changes to specific sites within the protein.

During titrations with sPGN, we did not observe significant chemical shift perturbations in Loop 2, where most C-type lectins have been shown to bind their carbohydrate ligands. However, we detected small but significant (\(\Delta\delta > 0.04\) ppm) dose-dependent chemical shift changes in five backbone amides within Loop 1: G112, T113, E114, W117, and E118 (Fig. 2A, B). Loop 1 has not previously been ascribed any function in ligand recognition by C-type lectins.

Inspection of the HIP/PAP primary sequence revealed that HIP/PAP E114 is part of a Loop 1 EPN sequence that is conserved in HIP/PAP and RegIIIy but is absent from Loop 1 of C-type lectins that recognize mannos and GlcNAc in a Ca\(^{2+}\)-dependent manner (Fig. 2C). Cross-peaks from the N116 side-chain amide also shifted significantly with titration of sPGN (Fig. 2B), consistent with this group serving as a critical hydrogen-bond donor to the sugar 3'-OH group in other lectins (7). Because proline residues lack backbone amides, PI115 is not detectable in the \(^{15}N/H\) HSQC spectrum.

In C-type lectins that require Ca\(^{2+}\) to bind carbohydrate, the glutamic acid and asparagine residues of EPN function to coordinate Ca\(^{2+}\) (6). Notably, the peptide bond between the E and P residues of the EPN motif of MBP undergoes a reversible switch from a trans to cis conformer in the presence of Ca\(^{2+}\), orienting the protein backbone for Ca\(^{2+}\) coordination and carbohydrate binding (9). Because RegIII lectins do not require Ca\(^{2+}\) to bind peptidoglycan, chitin, or mannan (1, 8), we asked whether the HIP/PAP E-P bond also isomerizes in the presence of Ca\(^{2+}\). The HIP/PAP \(^{15}N/H\) HSQC spectrum revealed no perturbation of either the E114 or N116 cross-peaks upon Ca\(^{2+}\) addition, indicating that the E-P peptide bond does not isomerize in the presence of Ca\(^{2+}\) (Fig. 2D). To determine the E-P peptide bond
configuration, we performed C(CO)NH TOCSY on $^{13}$C/$^{15}$N-labeled HIP/PAP to obtain the P115 $^{15}$Cj and $^{13}$Cy chemical shifts. The difference between these chemical shifts reports on the configuration of the peptide bond preceding the proline and was measured at 5.64 ppm in the absence of Ca$^{2+}$, indicative of the $trans$ conformer (10) (Fig. 2E). Collectively, these findings suggested that the EPN sequence might be important for ligand recognition in the RegIII lectins, but with a unique Ca$^{2+}$-independent functionality.

We fitted the Loop 1 chemical shift perturbations in the presence of sPGN to extract a binding affinity of 400 µM (Table 1). This $K_d$ is several orders of magnitude higher (~$10^4$-fold) than the $K_d$ of 26 nM for insoluble native peptidoglycan (1), suggesting a molecular basis for selective binding of HIP/PAP to bacterial cell surfaces despite the presence of soluble peptidoglycan fragments from the intestinal microbiota.

**HIP/PAP E114 is Essential for Peptidoglycan Binding and Bactericidal Activity.** To test the functional importance of HIP/PAP Loop 1 residues in peptidoglycan binding, we introduced conservative Glu (E) to Gln (Q) mutations into E114 and E118, the two Loop 1 residues with the most pronounced chemical shift perturbations upon sPGN titration (Fig. 3A). Binding of these mutants to sPGN was assessed by monitoring changes in their $^{15}$N/$^1$H HSQC spectra upon sPGN titration. Both mutants retained the same general chemical shift patterns of wild-type HIP/PAP, indicating that the overall protein structure remained intact (Fig. S1). The E118Q mutation had only a minor effect on sPGN binding affinity whereas the E114Q mutation weakened the interaction by $>5$-fold (Fig. 3A, Table 1). Thus, E114 plays a key role in peptidoglycan binding, suggesting the importance of the Loop 1 EPN motif in HIP/PAP peptidoglycan recognition.

We next assessed whether the reduced peptidoglycan binding of the E114 mutant correlated with HIP/PAP antimicrobial activity. HIP/PAP-E114Q showed a $>6$-fold reduction in bactericidal activity against *Staphylococcus aureus* (Fig. 3B), indicating that E114 is also critical for HIP/PAP bactericidal function. Surprisingly, we detected a similar reduction in bactericidal activity in HIP/PAP-E118Q (Fig. 3B). Thus, while E118 is not essential for peptidoglycan binding, it is important for antimicrobial function. Together, these data establish the importance of Loop 1 residues, including E114, for HIP/PAP bactericidal function.

**HIP/PAP E114 is Essential for Binding to the Peptidoglycan Carbohydrate Moiety.** Because the EPN motif is important for carbohydrate binding in other C-type lectins, we postulated that residues of the HIP/PAP Loop 1 motif specifically recognize the peptidoglycan carbohydrate moiety. To test this idea we examined HIP/PAP binding to chitooligosaccharides, β1,4GlcNAc polysaccharides that mimic the linear structure of the peptidoglycan backbone (Fig. S2). Titration of chitopentaose ($β1,4$GlcNAc)$_3$ into $^{15}$N-labeled HIP/PAP yielded chemical shift changes in several of the same Loop 1 residues (Fig. 4A) and along the same trajectory (Fig. 4B) as for sPGN. These changes included perturbations in both the E114 backbone amide and the N116 side-chain amide peaks (Fig. 4B). This suggests that similar chemical moieties in sPGN and chitopentaose elicit similar conformational changes in the protein, particularly at residues that are components of the Loop 1 EPN motif. The chitopentaose $K_d$ was 5.0 mM, indicating a moderate affinity interaction that is consistent with the millimolar $K_d$ typically observed for C-type lectin interactions with monosaccharides and short oligosaccharides.

![Fig. 3.](image)

**Table 1. Binding of PGN analogs to HIP/PAP mutants**

<table>
<thead>
<tr>
<th>HIP/PAP variant</th>
<th>sPGN $K_d$ (mM)*</th>
<th>Chitopentaose $K_d$ (mM)</th>
<th>Tri-DAP $K_d$ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>0.4 ± 0.2</td>
<td>5.0 ± 1.6</td>
<td>6.4</td>
</tr>
<tr>
<td>E114Q</td>
<td>2.1 ± 1.6*</td>
<td>NB</td>
<td>8.4</td>
</tr>
<tr>
<td>E118Q</td>
<td>0.6 ± 0.3</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>E114Q/E118Q</td>
<td>2.6 ± 1.0*</td>
<td>NB</td>
<td>8.0</td>
</tr>
</tbody>
</table>

* $K_d$ is calculated based on chemical shifts of eight Loop1 residues (±SEM).

* $p < 0.0002$.

* $p < 0.0003$.

![Fig. 4.](image)
(11, 12). Furthermore, the moderate $K_d$ for a soluble peptidoglycan analog is consistent with HIP/PAP's need to selectively recognize the bacterial cell surface without competitive inhibition by soluble peptidoglycan fragments from the intestinal microbiota. Addition of GlcNAc monosaccharides up to 20 mM produced no detectable chemical shift perturbations (Fig. 4B). Chito- 

We were unable to examine binding of chitohexaose or other longer chito-oligosaccharide chains due to their reduced solubility at millimolar concentrations. Binding was specific for chito-oligosaccharides, as titration of cellopentaose to 20 mM produced chemical shift perturbations that were too small to allow accurate determination of binding affinity (Fig. 4B). We also contained a Loop 1 EPN tripeptide. In contrast, RegIII each $\beta$- and RegIII each $\gamma$-type interactions are not predominant in HIP/PAP binding to sPGN, which includes fragments with longer saccharide chains (Fig. S3). Rather, our NMR and mutagenesis studies identify carbohydrate as the principal determinant of the HIP/PAP-peptidoglycan binding interaction.

**Carbohydrate is the Principal Determinant of the HIP/PAP-Peptidoglycan Interaction.** The peptidoglycan carbohydrate backbone is cross-linked by peptide moieties. To determine whether peptide might contribute to HIP/PAP recognition of peptidoglycan, we examined binding of chemically defined peptidoglycan structural analogs that contain peptide. GMDP (GlcNAc-MurNAc-L-Ala- D-Gln) consists of a disaccharide moiety corresponding to the repeating saccharide subunit of peptidoglycan but with a truncated peptide (Fig. S2). Because the disaccharide did not produce detectable chemical shift changes in the NMR binding assay (Fig. 4B), we reasoned that analysis of the NMR behavior of GMDP would allow us to identify peptide-specific alterations in the HIP/PAP 15N/1H HSQC spectrum. Surprisingly, GMDP titrations produced a pattern of chemical shift changes that were similar to those produced by sPGN, occurring at the same Loop 1 residues (Fig. 5 and Fig. S4).

While GMDP induced chemical shift changes along the same vector as sPGN and chitopentaose, they were on the opposite trajectory (Fig. 5 and Fig. S5). This behavior indicates a fast interconversion (microseconds or faster) of Loop 1 between two conformations in the apo state, with ligand binding shifting the equilibrium more strongly towards either conformer. To distinguish between the opposing chemical shift changes, we have designated the trajectory observed for chitopentaose as “Type I” and that observed for GMDP as “Type II” (Fig. 5). Other chemically defined peptide-containing ligands, including muramyl dipeptide (MDP), muramyl tripeptide, and trachael cytoxoxin [a naturally occurring disaccharide-tetrapeptide fragment of DAP-type peptidoglycan (13)] also induced Type II perturbations in the same Loop 1 residues as GMDP. All chemically defined peptide-containing ligands tested bound with low millimolar $K_d$ (Table S1). These results identify Type II chemical shift trajectories as a hallmark of peptide interactions with HIP/PAP, sPGN titration elicits only Type I chemical shift trajectories (Fig. 2B), suggesting that carbohydrate interactions predominate during HIP/PAP binding to sPGN.

Titrations of the pure peptide ligand Tri-DAP into the HIP/ 
PAP-E114Q mutant yielded a $K_d$ of 8.4 mM, similar to the $K_d$ of 6.4 mM calculated for the wild-type protein (Table 1). Likewise, binding to HIP/PAP-E114Q/E118Q produced a $K_d$ of 8.0 mM. This establishes that HIP/PAP E114 and E118 are dispensable for binding to the peptidoglycan peptide moiety and suggests that the peptide interacts with HIP/PAP at a distinct site and elicits conformational changes at these Glu residues through an indirect mechanism.

To further explore the relative importance of HIP/PAP interactions with peptide, we examined 15N/1H HSQC spectra obtained in the presence of chemically defined peptide-containing ligands, comparing them to spectra obtained during titrations of the saccharide ligand chitopentaose (Fig. S6). These comparisons revealed several peptide-specific chemical shift changes in the region of the 15N/1H HSQC spectrum corresponding to Asn and Gln side-chain amides (Fig. S6). Low crosspeak intensities in triple resonance datasets precluded us from making definitive assignments of these side-chain resonances; however, the peptide-specific alterations in the 15N/1H HSQC spectrum represent another set of unique hallmarks of peptide interactions with HIP/PAP. Notably, none of these peptide-specific alterations was evident upon sPGN titration, even at saturating ligand concentrations (Fig. S6). This supports the idea that peptide interactions are not predominant in HIP/PAP binding to sPGN, which includes fragments with longer saccharide chains (Fig. S3).

**Predicting Peptidoglycan Binding Activity of Other RegIII Family Members.** We compared the HIP/PAP sequence to that of other RegIII family members to determine whether the presence of a Loop 1 EPN predicts peptidoglycan binding. RegIIIy and RegIIIC each also contain a Loop 1 EPN tripeptide. In contrast, RegIIb lacks this motif, substituting Gln (Q) for Gln (E) at the first position in the tripeptide (Fig. 5). We have previously shown that RegIIb
Determination of a high resolution structure of HIP/PAP in the Loop 1 EPN-carbohydrate interaction could be due to the motif that coordinates Ca$^{2+}$-dependent saccharide binding, peptidoglycan recognition, and bacterial killing. Further, the presence of an EPN tripeptide was shown to elucidate the exact mechanism by which the HIP/PAP EPN motif supports Ca$^{2+}$-independent carbohydrate binding. Nevertheless, our findings suggest that the EPN tripeptide is a flexible motif that plays multiple roles in lectin-carbohydrate interactions.

A limited number of other CTLD-containing proteins also recognize carbohydrates in a Ca$^{2+}$-independent manner via mechanisms that are distinct from HIP/PAP. Dectin-1 binds to oligomers of the fungal cell wall saccharide β,3-glucan. Unlike HIP/PAP, dectin-1 does not have a Loop 1 EPN motif, and binding to β-glucan involves interactions with the β3 strands of dectin-1 dimers (14). The C-type lectin langerin binds mannose in a Ca$^{2+}$-independent manner but does so via a Loop 2 site that is secondary to a canonical Ca$^{2+}$-dependent binding site (15).

HIP/PAP carries out its bactericidal functions in the intestinal lumen, an environment rich in soluble peptidoglycan fragments derived from resident bacteria. Because HIP/PAP bactericidal activity requires recognition of intact bacteria (1), high-affinity recognition of soluble peptidoglycan fragments would be counterproductive by competitively inhibiting cell wall binding. Our finding that HIP/PAP binds to its native polymeric ligand with a much higher affinity than to soluble fragments provides a molecular explanation for the ability of HIP/PAP to selectively bind intact bacteria in the luminal environment.

A low affinity for soluble analogs is a characteristic feature of lectins that bind to linear polysaccharides with repeated epitopes. For example, mucin-binding lectins bind to their native extended ligands with affinities up to $10^6$-fold higher than for the corresponding monovalent carbohydrates that are bound with millimolar $K_d$ values (12). Thermodynamic studies have elucidated the mechanism by which mucin-binding lectins achieve selective high-affinity binding to extended native ligands, showing that the lectins "bind and slide" from carbohydrate to carbohydrate epitope, resulting in a gradient of decreasing microaffinity constants. This "recapture effect" results in a decreased off-rate and an increased apparent binding affinity (16). Thus, a hallmark feature of these interactions is a direct relationship between binding affinity and carbohydrate chain length. Our finding that the strength of HIP/PAP binding depends on saccharide chain length strongly suggests that HIP/PAP utilizes a similar binding mechanism. Therefore, we propose that dynamic recognition of the multivalent carbohydrate backbone of native peptidoglycan is an essential mechanism governing selective high-affinity interactions between HIP/PAP and the bacterial cell wall.

The molecular basis of HIP/PAP recognition of peptidoglycan differs fundamentally from that of the peptidoglycan recognition protein family (PGRPs). Drosophila PGRPs bind with high affinity to soluble PGN fragments shed by bacteria, initiating signaling cascades that direct expression of antibacterial peptides. Peptidoglycan recognition by PGRPs involves moderate- to high-affinity interactions with the peptide moiety, allowing discrimination between Gram-positive and Gram-negative bacteria and the initiation of immune responses specific for either group (17). HIP/PAP, in contrast, interacts with the carbohydrate moiety of peptidoglycan, accomplishing high-affinity binding to the bacterial cell wall through multivalent interactions with the highly clustered presentation of carbohydrate epitopes.

Discussion

Here, we have used solution NMR to gain insight into the molecular basis for peptidoglycan recognition by HIP/PAP, a bactericidal C-type lectin. Surprisingly, we found that Ca$^{2+}$-independent recognition of the peptidoglycan carbohydrate backbone involves an EPN motif similar to that required for Ca$^{2+}$-dependent recognition of mannose and GlcNAc-containing saccharides by C-type lectins such as MBP. Mutation of the Glu residue (E114) of this tripeptide revealed the functional importance of this site in HIP/PAP saccharide binding, peptidoglycan recognition, and bacterial killing. Further, the presence of an EPN tripeptide correlates with peptidoglycan binding activity among mouse and human RegII family lectins.

Ca$^{2+}$-dependent saccharide recognition by lectins such as MBP and DC-SIGN is critically dependent on a Loop 2 EPN motif that coordinates Ca$^{2+}$ and forms hydrogen bonds with sugar hydroxyls (6, 5). The EPN sequence is an essential determinant of specificity for GlcNAc and mannose, which are related by the orientations of their 3- and 4-OH groups (5). Given that HIP/PAP also selectively recognizes GlcNAc and mannose polysaccharides (1), we propose that the HIP/PAP Loop 1 EPN motif may govern saccharide specificity. The loss of Ca$^{2+}$ dependence in the Loop 1 EPN-carbohydrate interaction could be due to the absence of additional Ca$^{2+}$ coordination sites such as the ND dipeptide or the constitutive trans conformer of the EPN proline. Determination of a high resolution structure of HIP/PAP in complex with a peptidoglycan structural analog will be required to elucidate the exact mechanism by which the HIP/PAP EPN motif supports Ca$^{2+}$-independent carbohydrate binding.
for 30 min. The suspension was heated to 90 °C for 30 min, cooled to 37 °C, and lysostaphin (Sigma) added to 50 μg/mL. After overnight incubation at 37 °C, the preparation was heated to 95 °C for 5 min and centrifuged for 10 min at 10,000 g. The soluble fraction was lyophilized, resuspended in 1 mL milliQ H₂O and desalted on a Sephadex G-10 column equilibrated in 25 mM MES pH 5.5, 25 mM NaCl. The sPGN preparation was characterized by size exclusion chromatography (Fig. S3), and total fragment concentrations were estimated by quantifying carbohydrate reducing termini (19). Average fragment chain lengths were determined from the molar ratio of carbohydrate (determined by absorbance at 218 nm) to reducing termini.

Chemically Defined Ligands. Chito-oligosaccharides (Seikagaku), cellobiopentaose (Seikagaku), GMDP (Calbiochem), GlcNAc (Sigma), and Tri-DAP (InvivoGen) were obtained from commercial sources.

NMR Spectroscopy. To prepare samples for NMR, Escherichia coli BL21-CodonPlus (DE3)-RILP transformed with expression plasmids were grown to prepare samples for NMR, were obtained from commercial sources.


Fig. S1. Characterization of HIP/PAP mutant proteins. $^{15}$N/$^1$H HSQC spectra overlays of wild-type HIP/PAP, and E114Q, and E118Q mutants, establishing that the mutations do not alter overall protein structure.
Fig. S2. Structures of peptidoglycan derivatives used in solution NMR binding studies. mDAP indicates meso-diaminopimelic acid.
Fig. S3. Characterization of solubilized peptidoglycan. (A) Solubilized peptidoglycan (sPGN) was generated by sonication and lysostaphin digestion of insoluble Staphylococcus aureus peptidoglycan purified as described in Materials and Methods. The sPGN was loaded onto a 1.5 × 70 cm Sephacryl S-100 column for analysis by size-exclusion chromatography. Individual fractions were assayed for carbohydrate content by absorbance at 218 nm. Peak fractions were pooled as indicated, and assayed for moles of total carbohydrate by absorbance at 218 nm and moles of reducing termini as described (1). The percentage of reducing termini in each pooled peak (relative to the reducing termini in total solubilized peptidoglycan) is indicated. Average chain lengths for pooled fractions in peaks 1–3 were calculated from the ratio of total carbohydrate content to moles of reducing termini. Peaks 4–6 contained very low concentrations of reducing termini, suggesting the presence of anhydro groups or otherwise altered or damaged reducing termini. Void volume ($V_0$) and total column bed volume ($V_t$) are indicated. (B) Peaks 2 and 3 were pooled, concentrated, and analyzed for binding to $^{15}$N-labeled HIP/PAP by NMR in comparison to total sPGN. Chemical shift perturbations at E114 in the presence of 1.3 mM ligand are shown. We fitted the chemical shift perturbations at E114 to extract a $K_d$ of 1.4 mM for the 12 saccharide fraction. Although we did not have sufficient material to titrate the 5 saccharide fraction to saturation, our results show that the $K_d$ is greater than 3 mM, with a projected $K_d$ of 4.9 mM (calculated by NMRView). These results support the idea that the affinity of the HIP/PAP-peptidoglycan interaction is governed by carbohydrate chain length.

Fig. S4. GMDP induces conformational changes in HIP/PAP Loop 1. Quantification of HIP/PAP chemical shift changes from the $^{15}$N/$^1$H HSQC in the presence of 13 mM GMDP.

Fig. S5. Chitopentaose and GMDP induce distinct chemical shift trajectories in Loop 1 residues. Superimposed $^{15}$N/$^1$H HSQC spectra of $^{15}$N-labeled HIP/PAP with titrated GMDP and chitopentaose. Several switch residues are indicated, showing that chitopentaose and GMDP elicit chemical shift changes on the same vector but with opposing trajectories. Zoomed views of cross-peaks representing the E114 backbone amide and the N116 side-chain amide (N116 Nδ2) are shown in Fig. 5.
Fig. S6. $^{15}$N/$^1$H HSQC overlays showing that peptide-specific cross-peaks are not induced by sPGN. We examined $^{15}$N/$^1$H HSQC spectra obtained in the presence of the peptide-containing ligands GMDP, MDP, and Lys-type tetrapeptide (L-Ala-D-Glu-L-Lys-D-Ala), comparing them to spectra obtained with titration of the pure saccharide ligand chitopentaose. These comparisons revealed the appearance of several new peptide-specific cross-peaks (indicated by arrows) on the slow exchange timescale in the region of the $^{15}$N/$^1$H HSQC spectrum corresponding to Asn and Gln side-chain amides. Importantly, these peptide-specific cross-peaks are absent from $^{15}$N/$^1$H HSQC spectra obtained in the presence of saturating sPGN, supporting the conclusion that carbohydrate is the principal determinant of the HIP/PAP-peptidoglycan interaction.
Fig. S7. HIP/PAP E114 and E118 are not essential for recognition of the peptidoglycan peptide moiety. Tri-DAP was titrated into $^{15}$N-labeled wild-type HIP/PAP and HIP/PAP-E114Q and HIP/PAP-E114Q/E118Q mutants. Similar dose-dependent chemical shift perturbations were observed in all three proteins. As E114 is essential for recognition of chitopentaose (Fig. 4C), these results demonstrate that E114 is necessary for HIP/PAP recognition of peptidoglycan carbohydrate but not the peptide moiety.
Table S1. HIP/PAP binding to soluble PGN analogs

<table>
<thead>
<tr>
<th>Analog</th>
<th>$K_d$ (mM)</th>
<th>Chemical shift trajectory</th>
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<tbody>
<tr>
<td>sPGN†</td>
<td>0.4 ± 0.2</td>
<td>Type I</td>
</tr>
<tr>
<td>sPGN (12 saccharide fraction)</td>
<td>1.4 ± 0.4</td>
<td>Type I</td>
</tr>
<tr>
<td>sPGN (5 saccharide fraction)</td>
<td>4.9 ± 1.3*</td>
<td>Type I</td>
</tr>
<tr>
<td>GlcNAc</td>
<td>NB</td>
<td></td>
</tr>
<tr>
<td>Chitobiose</td>
<td>NB</td>
<td></td>
</tr>
<tr>
<td>Chitotriose</td>
<td>nd</td>
<td>Type I</td>
</tr>
<tr>
<td>Chitopentaose</td>
<td>5.0 ± 1.6</td>
<td>Type I</td>
</tr>
<tr>
<td>Cellpentaoese</td>
<td>NB</td>
<td></td>
</tr>
<tr>
<td>GMDP</td>
<td>11.0 ± 4.5</td>
<td>Type II</td>
</tr>
<tr>
<td>MDP</td>
<td>8.6 ± 3.4</td>
<td>Type II</td>
</tr>
<tr>
<td>MDP-D,D</td>
<td>28 ± 5</td>
<td>Type II</td>
</tr>
<tr>
<td>MTri-DAP</td>
<td>5.5</td>
<td>Type II</td>
</tr>
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<td>Tri-DAP</td>
<td>6.4</td>
<td>Type II</td>
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<td>Tri-Lys</td>
<td>8.8</td>
<td>Type II</td>
</tr>
<tr>
<td>TCT</td>
<td>7.1</td>
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NB, no binding detected; nd, not determined; ±SEM reported.

We assessed HIP/PAP binding to several chemically defined peptide-containing analogs. MDP (Sigma) produced a similar chemical shift pattern as seen for GMDP, with perturbations occurring along the Type II trajectory. An MDP variant (MDP-D,D; InvivoGen), in which the chirality of the α-carbon of the first amino acid (Ala) is altered from the usual L-form to a D-form, bound with ~3-fold reduced affinity, indicating sensitivity to peptide conformation.

We also examined binding of peptidoglycan analogs with longer peptide chains that more closely resemble the native peptidoglycan structure. Muramyl tripeptide (MTri-DAP; InvivoGen) is a disaccharide tripeptide in which the third amino acid in the chain is meso-diaminopimelic acid (mDAP; Fig. 5A). MTri-DAP and the related analog Tri-DAP (InvivoGen), which consists solely of peptide, each induced Type II shifts in the same Loop 1 residues as GMDP, as well as significant chemical shift perturbations over a wider range of residues (Fig. 5B). Tracheal cytotoxin, a naturally occurring disaccharide-tetrapeptide fragment of DAP-type peptidoglycan, likewise produced Type II chemical shifts and a low millimolar $K_d$ (TCT was isolated as previously described (1)). Additionally, we observed evidence of cooperative binding for tripeptide analogs (Fig. 5B), but were unable to determine accurate $K_d$s for low affinity binding sites due to ligand solubility limits; thus we report $K_d$s for the high affinity binding sites for MTri-DAP and Tri-DAP. Neither binding affinity nor chemical shift trajectory was significantly altered by substitution of L-Lys at the third amino acid position of the stem peptide.

$K_d$s were calculated on the basis of chemical shift perturbations at eight Loop 1 residues, and we have reported the average and standard error of the 8 individual $K_d$s.


†Average saccharide chain length = 36 saccharides.
‡Projected $K_d$ based on titration to 3.2 mM.