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.

antimicrobial protein | C-type lectin | intestine | nuclear magnetic resonance | bacterial cell wall

The 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.

The bactericidal activities of RegIIIγ and HIP/PAP depend on binding to cell wall peptidoglycan, a polymer of alternating N-acetylglucosamine (GlcNAc) and N-acetylmuramic acid (MurNAc) cross-linked by short peptides. This finding identified RegIII lectins as a distinct class of peptidoglycan binding proteins (1). However, given the lack of canonical carbohydrate binding motifs in RegIII proteins, the molecular basis for lectin-mediated peptidoglycan recognition remains unknown. Furthermore, RegIII lectins are secreted into the intestinal lumen where there are abundant soluble peptidoglycan fragments derived from the resident microbiota through shedding or enzymatic degradation of the bacterial cell wall. Thus, it remains unclear how RegIII lectins selectively bind to bacterial surfaces without competitive inhibition by soluble peptidoglycan fragments in the lumenal environment.

In this study, we use solution NMR spectroscopy to understand the molecular basis of peptidoglycan recognition by HIP/PAP. First, we identify a HIP/PAP "EPN motif" that is essential for recognition of the peptidoglycan carbohydrate backbone and is required for efficient bacterial killing. Whereas EPN tripeptides are involved in Ca^{2+}-dependent recognition of carbohydrates by other C-type lectins (5, 6), the HIP/PAP EPN sequence differs in that it supports Ca^{2+}-independent saccharide recognition and is found in an unusual location in the protein. Second, we show that HIP/PAP binding affinity to soluble peptidoglycan analogs depends on carbohydrate chain length, pointing to a model in which multivalent presentation of carbohydrate epitopes in native peptidoglycan governs high-affinity interactions between HIP/PAP and the bacterial cell surface.

Results

RegIII Family Members Lack Canonical Carbohydrate Binding Motifs. The three-dimensional structure of HIP/PAP exhibits a C-type lectin fold with its characteristic "long loop" structure (7). This loop exhibits two distinct subdomains, which we have designated "Loop 1" (residues 107–121) and "Loop 2" (residues 130–145) (Fig. 1*A*). In other C-type lectins, such as mannose binding protein (MBP) and DC-SIGN, a tripeptide motif in Loop 2 (EPN or QPD) is critical for Ca^{2+}-dependent binding to saccharides, interacting with both Ca^{2+} and carbohydrate to coordinate the sugar 3-OH (6) (Fig. 1*B*). A second motif (ND) located in the β4 strand also makes contacts with both Ca^{2+} and the 4-OH

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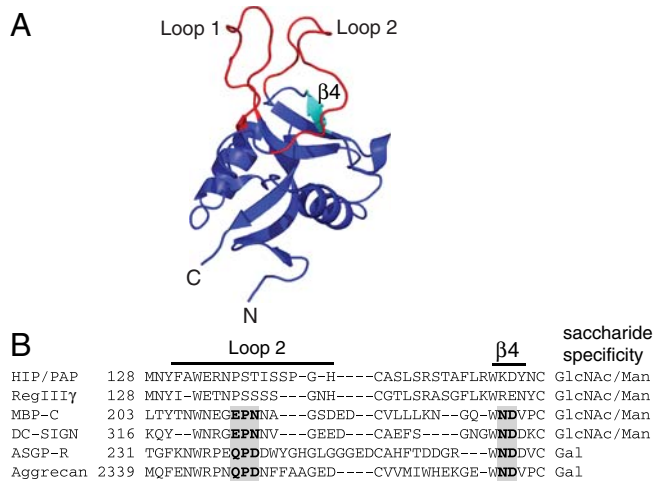

Fig. 1. HIP/PAP lacks canonical C-type lectin-carbohydrate binding motifs. (*A*) NMR structure of HIP/PAP (PDB code 2GO0.pdb) (22), with *Loops 1* and *2* of the long loop region in *Red* and the β4 strand in *Blue*. (*B*) Alignment of the long loop region of RegIII family members with other C-type lectin family members. MBP-C and DC-SIGN harbor *Loop 2* EPN motifs that govern sugar ligand binding and confer selectivity for mannose (Man) and GlcNAc (5). Asialoglycoprotein receptor (ASGP-R) and aggrecan contain *Loop 2* QPD motifs that confer selectivity for Gal and GalNAc. Despite their selective binding to GlcNAc and Man polysaccharides (1), RegIII lectins lack the *Loop 2* EPN motif.

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 EPN motif binding GlcNAc or mannose (which have equatorial 3- and 4-OH groups), and the QPD motif binding GalNAc or galactose (which have axial 4-OH groups) (5) (Fig. 1*B*). While RegIIIγ 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 β4 ND motifs (Fig. 1*B*), 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 $^{15}N/^{1}H$ 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 $^{15}N/^{1}H$ HSQC spectra. As we had previously assigned the backbone chemical shifts of 98% of the HIP/PAP $^{15}N/^{1}H$ 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. 2*A*, *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 RegIIIγ but is absent from Loop 1 of C-type lectins that recognize mannose and GlcNAc in a Ca^{2+}-dependent manner (Fig. 2*C*). Cross-peaks from the N116 side-chain amide also shifted significantly with titration of sPGN (Fig. 2*B*),

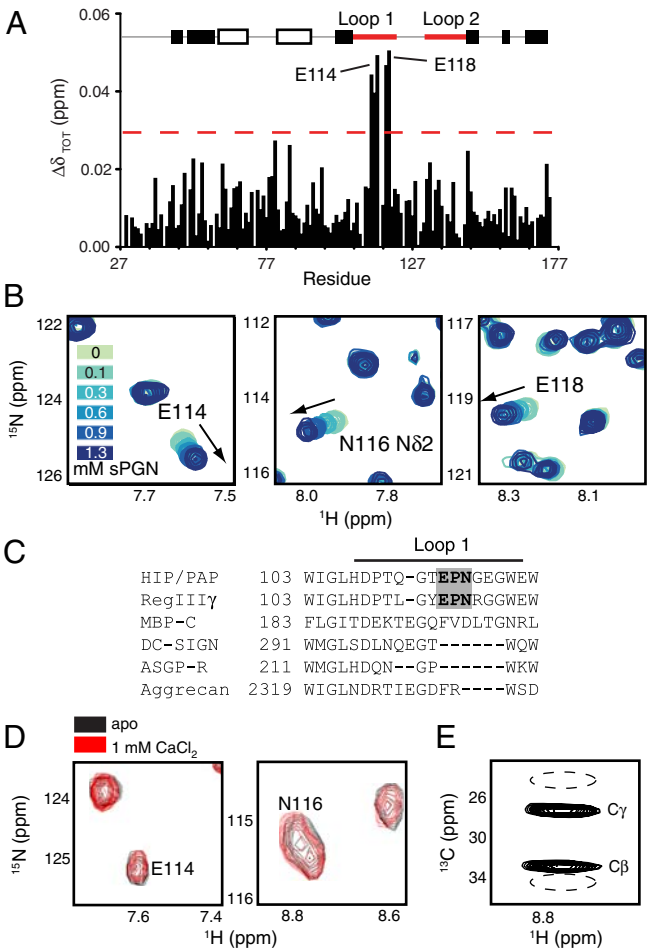

Fig. 2. Peptidoglycan induces HIP/PAP *Loop 1* conformational changes that encompass a conserved EPN motif. (*A*) Quantification of HIP/PAP chemical shift changes from the $^{15}N/^{1}H$ HSQC in the presence of 1.3 mM solubilized *Staphylococcus aureus* peptidoglycan (sPGN). The *Red Dotted Line* indicates chemical shift changes greater than the mean $+2\sigma$ (0.029 ppm). (*B*) $^{15}N/^{1}H$ HSQC spectra of 100 μM ^{15}N-HIP/PAP titrated with increasing concentrations of sPGN. Nδ2, side-chain amide. (*C*) E114 is part of a HIP/PAP *Loop 1* EPN motif that is conserved in RegIIIγ but is lacking in Ca^{2+}-dependent C-type lectins. (*D*) $^{15}N/^{1}H$-HSQC spectra of 100 μM ^{15}N-HIP/PAP in the absence and presence of 1 mM $CaCl_2$. The lack of chemical shift perturbations indicates no isomerization of the E-P peptide bond in the presence of Ca^{2+}. (*E*) C(CO)NH TOCSY strip of $^{13}C/^{15}N$-HIP/PAP P115. The separation between the Cγ and Cβ peaks is 5.64 ppm, which is within the expected range for a *trans* peptide bond (10). *Dashed Circles* indicate the cross-peak locations predicted for a *cis* peptide bond.

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, P115 is not detectable in the $^{15}N/^{1}H$ 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 $^{15}N/^{1}H$ 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. 2*D*). 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 $^{13}C\beta$ and $^{13}C\gamma$ 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. 2*E*). 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. 3*A*). 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. 3*A*, 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 *Listeria monocytogenes* (Fig. 3*B*), 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. 3*B*). 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 EPN 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 carbohydrate backbone (Fig. S2). Titration of chitopentaose $(\beta 1,4\text{GlcNAc})_5$ into ^{15}N-labeled HIP/PAP yielded chemical shift changes in several of the same Loop 1 residues (Fig. 4*A*) and along the same trajectory (Fig. 4*B*) as for sPGN. These changes

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

HIP/PAP variant	sPGN K_d (mM)*	Chitopentaose K_d (mM)	Tri-DAP K_d (mM)
Wild-type	0.4 ± 0.2	5.0 ± 1.6	6.4
E114Q	2.1 ± 1.6†	NB	8.4
E118Q	0.6 ± 0.3	nd	nd
E114Q/E118Q	2.6 ± 1.0‡	NB	8.0

NB, no binding detected; nd, not determined

*K_ds were calculated based on chemical shift changes of eight Loop1 residues (±SEM).

†P < 0.0002.

‡P < 0.0003.

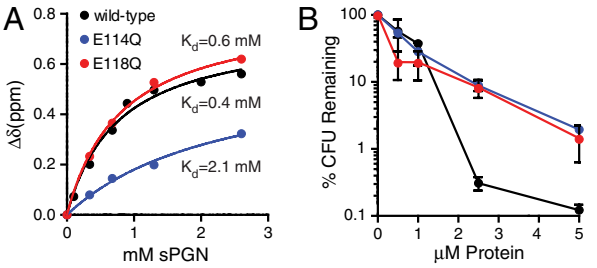

Fig. 3. HIP/PAP E114 is essential for peptidoglycan binding and bactericidal activity. (*A*) *Staphylococcus aureus* sPGN titrated into 100 μM ^{15}N-HIP/PAP Loop 1 mutants. HIP/PAP-E114Q has a reduced affinity for sPGN, as evidenced by a decrease in the dose-dependent chemical cross-peak perturbation of T113 in the E114Q mutant. K_ds were derived from chemical shift changes at eight Loop 1 residues and averaged (values ± SEM are also reported in Table 1). (*B*) Point mutations in HIP/PAP Loop 1 attenuate bactericidal activity. *Listeria monocytogenes* was incubated with purified wild-type or mutant HIP/PAP for 2 h at 37 °C and quantified by dilution plating. Assays were done in triplicate. Mean ± SEM is plotted.

included perturbations in both the E114 backbone amide and the N116 side-chain amide peaks (Fig. 4*B*). 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_ds typically observed for C-type lectin interactions with monosaccharides and short oligosaccharides

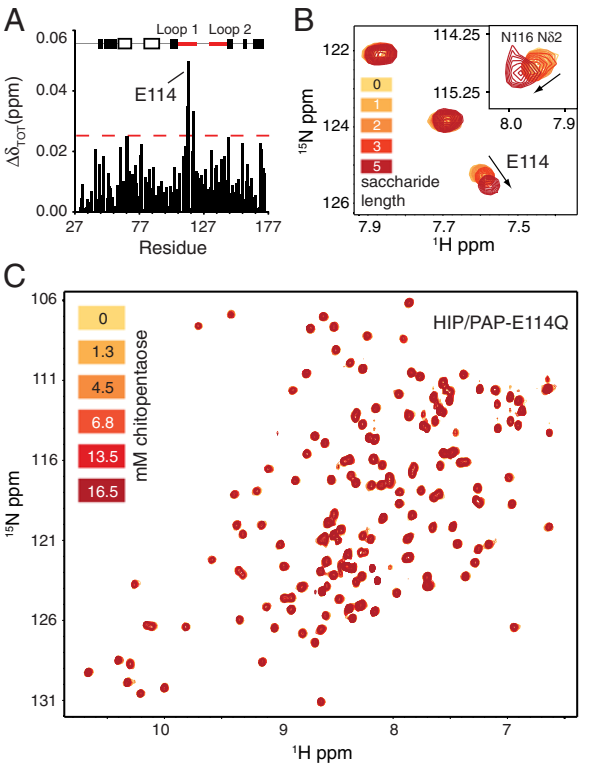

Fig. 4. Carbohydrate recognition by the HIP/PAP Loop 1 EPN motif depends on saccharide chain length. (*A*) Quantification of HIP/PAP chemical shift changes from the $^{15}N/^{1}H$ HSQC recorded in the presence of 20 mM chitopentaose. The *Red Dotted Line* indicates chemical shift perturbations greater than the mean $+2\sigma$ (0.024 ppm). (*B*) Overlay of residue E114 backbone amide and N116 side-chain amide chemical shifts with addition of 20 mM GlcNAc, chitobiose, chitotriose, or chitopentaose. (*C*) Mutation of HIP/PAP E114 abolishes chitopentaose binding. Superimposed $^{15}N/^{1}H$ HSQC spectra of ^{15}N-labeled HIP/PAP-E114Q with titrated chitopentaose, showing an absence of chemical shift perturbations in the mutant protein.

(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. 4*B*). Chitobiose $(\beta 1,4\text{-GlcNAc})_2$ also failed to elicit significant chemical shift changes, whereas chitotriose $(\beta 1,4\text{-GlcNAc})_3$ titration up to 20 mM produced chemical shift perturbations that were too small to allow accurate determination of binding affinity (Fig. 4*B*). We were unable to examine binding of chitohexaose or other longer chitooligosaccharide chains due to their reduced solubility at millimolar concentrations. Binding was specific for chitooligosaccharides, as titration of cellopentaose $(\beta 1,4\text{-Glc})_5$ did not elicit any significant chemical shift changes. This accords with the absence of detectable HIP/PAP binding to insoluble cellulose, the source of cellopentaose (1). These results show that HIP/PAP Loop 1 residues, including E114 and N116, recognize soluble analogs of the peptidoglycan carbohydrate backbone, and demonstrate that binding affinity is dictated by polysaccharide chain length.

We next examined the relationship between HIP/PAP binding affinity and polysaccharide chain length for saccharide–peptide chains derived from native peptidoglycan. We fractionated sPGN by size exclusion chromatography and isolated two well-resolved peaks having average saccharide chain lengths of 5 and 12 sugars, respectively (Fig. S3*A*). Titrations into ^{15}N-labeled HIP/PAP revealed that the longer sPGN chains elicited more pronounced chemical shift perturbations at E114 as compared to the shorter chains (Fig. S3*B*). We extracted a K_d of 1.4 mM for binding of the 12 saccharide fraction and a K_d of >3 mM for binding of the 5 saccharide fraction (Fig. S3*A* and Table S1). By comparison, we calculated an average saccharide chain length of 36 for unfractionated sPGN that binds with a K_d of 0.4 mM. These results support the idea that the affinity of HIP/PAP binding to native peptidoglycan is governed by saccharide chain length.

We next tested whether E114 is essential for carbohydrate ligand binding. Chitopentaose titration up to 20 mM into the HIP/PAP-E114Q mutant yielded no detectable chemical shift perturbations (Fig. 4*C*), indicating a lack of binding. This demonstrates that E114 is critical for binding saccharides with structures that mimic the peptidoglycan carbohydrate backbone and establishes the importance of the HIP/PAP EPN motif in carbohydrate ligand recognition.

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. 4*B*), we reasoned that analysis of the NMR behavior of GMDP would allow us to identify peptide-specific alterations in the HIP/PAP ^{15}N/^{1}H 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

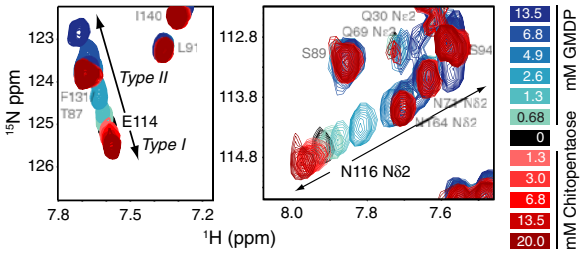

Fig. 5. Carbohydrate is the principal determinant of the HIP/PAP-peptidoglycan interaction. ^{15}N/^{1}H HSQC spectra overlay highlighting the opposing trajectories of E114 backbone amide and N116 side-chain amide chemical shift perturbations in response to titration with chitopentaose or GMDP. *Type I* and *II* chemical shift trajectories are indicated. sPGN induces only *Type I* trajectories (Fig. 2*B*), indicating that carbohydrate is the principal determinant of the HIP/PAP interaction with soluble native peptidoglycan.

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 tracheal cytotoxin [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*s* (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. 2*B*), suggesting that carbohydrate interactions predominate during HIP/PAP binding to sPGN.

Titration 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 ^{15}N/^{1}H 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 ^{15}N/^{1}H 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 ^{15}N/^{1}H 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). Rather, our NMR and mutagenesis studies identify carbohydrate as the principal determinant of the HIP/PAP-peptidoglycan binding interaction.

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. RegIIIγ and RegIIIβ each also contain a Loop 1 EPN tripeptide. In contrast, RegIIIα lacks this motif, substituting Gln (Q) for Glu (E) at the first position in the tripeptide (Fig. 6*A*). We have previously shown that RegIIIγ

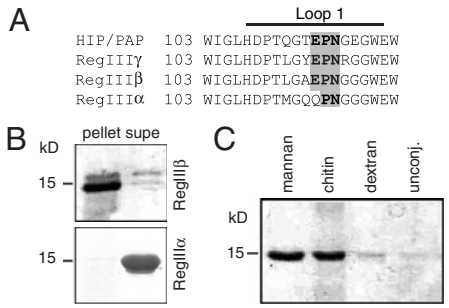

Fig. 6. The presence of a Loop 1 EPN motif predicts peptidoglycan binding in other RegIII family members. (*A*) Alignments of human and mouse RegIII family members showing a conserved EPN sequence in *Loop 1* of RegIIIγ and RegIIIβ but not RegIIIα. (*B*) RegIIIβ but not RegIIIα binds peptidoglycan. Ten μg of recombinant RegIIIβ or RegIIIα was added to 50 μg insoluble *Bacillus subtilis* peptidoglycan and pelleted. Pellet and supernatant (*Supe*) fractions were analyzed by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie blue staining. The lower molecular weight form of RegIIIβ in the pellet results from cleavage at an N-terminal trypsin site by a peptidoglycan-associated proteolytic activity (1, 4). (*C*) RegIIIβ selectively binds to GlcNAc and mannose polysaccharides. RegIIIβ was bound to immobilized polysaccharide using a previously described assay (1). After washing, bound proteins were released by boiling and analyzed by SDS-PAGE and Coomassie blue staining. Unconj., unconjugated Sepharose.

and HIP/PAP exhibit similar binding specificities, recognizing peptidoglycan, chitin, and mannan but not dextran (1, 8). We assessed the binding properties of RegIIIβ and RegIIIα in pull-down assays with insoluble peptidoglycan. RegIIIβ localized to the peptidoglycan pellet, indicating binding, while RegIIIα remained in the supernatant, showing no detectable peptidoglycan binding (Fig. 6*B*).

We next assayed the carbohydrate binding specificity of RegIIIβ. RegIIIβ was incubated with carbohydrates conjugated to Sepharose beads, including mannan, chitin, and dextran. RegIIIβ bound to mannan and chitin, but not dextran (Fig. 6*C*), exhibiting the same binding specificity as HIP/PAP and RegIIIγ (1, 8). Thus, peptidoglycan binding ability correlates with a Loop 1 EPN tripeptide in RegIII family proteins, and binding activities can be predicted by the presence of this motif.

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 RegIII 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 (5, 6). 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. 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 β1,3-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 lumenal 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_ds (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.

Materials and Methods

Expression and Purification of Recombinant Proteins. Pro-HIP/PAP was expressed and purified as previously described (4, 8). Point mutations were introduced using the QuikChange II Site Directed Mutagenesis Kit (Stratagene) and primers harboring the desired mutations. Expression and purification of mutant HIP/PAP was carried out as for the wild-type protein (4, 8). Expression and purification of RegIIIα and RegIIIβ were carried out as described for RegIIIγ (8).

Preparation of Solubilized Peptidoglycan (sPGN). Native peptidoglycan was isolated from *Staphylococcus aureus* as previously described (18). Insoluble PGN was resuspended to 10 mg/mL in 50 mM Tris-HCl pH 8 and sonicated

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 H_2O 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. Chitooligosaccharides (Seikagaku), cellopentaose (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 in M9 minimal media containing 1 g/L of $^{15}NH_4Cl$ for uniformly ^{15}N labeled samples, further substituting unlabeled glucose with 3 g/L of $^{13}C_6$-glucose for $^{15}N/^{13}C$-labeled samples. HIP/PAP was purified as described (8). NMR data were recorded at 25 °C using a cryoprobe-equipped Varian Inova 600 MHz spectrometer. Spectra were processed using NMRPipe (20) and analyzed with NMRView (21).

Antimicrobial Assays. Antimicrobial assays were done using HIP/PAP and HIP/PAP mutants engineered to lack the inhibitory N-terminal pro-segment (4). *Listeria monocytogenes* (strain EGD-e) was exposed to the indicated lectin concentrations at 37 °C for 2 h, and surviving bacteria were quantified by dilution plating as described (1).

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