Innate immunity: Bacterial cell-wall muramyl peptide targets the conserved transcription factor YB-1


Abstract

The bacterial cell wall muramyl dipeptides MDP and glucosaminyl-MDP (GMDP) are powerful immunostimulators but their binding target remains controversial. We previously reported expression cloning of GMDP-binding polypeptides and identification of V-box protein 1 (YB-1) as their sole target. Here we show specific binding of GMDP to recombinant YB-1 protein and subcellular colocalization of YB-1 and GMDP. GMDP binding to YB-1 upregulated gene expression levels of NF-κB2, a mediator of innate immunity. Furthermore, YB-1 knockout abolished GMDP-induced NFκB2 expression. GMDP/NFκB1 stimulation led to NFκB2 cleavage, transport of activated NFκB2 p52 to the nucleus, and upregulation of NFκB2-dependent chemokine CXCR4 gene expression. Therefore, our findings identify YB-1 as a new target for muramyl peptide signaling.

1. Introduction

Muramyl dipeptides MDP and GMDP are potent adjuvants and immunostimulators released from the cell wall of invading bacteria by the action of lysozyme and amidases, and synergize with lipopolysaccharide (LPS) in the induction of the innate immune response [1–3]. MDP is composed of N-acetylmuramic acid linked to the N-terminus of an ε-alanine d-isoglutamine dipeptide, whereas GMDP bears an additional N-terminal acetyl-glucosamine moiety. The two molecules have similar bioactivity and are thought to target the same binding sites and signaling pathways. Understanding the precise mechanisms underlying MDP/GMDP immunostimulatory effects is important for the management of immune and inflammatory disorders associated with dysregulation of innate immunity, including Crohn’s and inflammatory bowel diseases, atherosclerosis, arthritis, systemic lupus, and type 1 diabetes.

The precise molecular target(s) for peptidoglycan-derived molecules remain controversial. Most attention has focused on NOD (nucleotide oligomerization domain)-type pattern-recognition receptors (PRRs). NOD2, encoded by the CARD15 gene in human, has been proposed as a sensor for cell-wall peptidoglycans [4–6]. NOD2 protein is expressed in cells of the innate immune system including macrophages, polymorphonuclear neutrophils, and dendritic cells. These respond to NOD2 stimulation by the induction of nuclear factor (NF)-κB and cytokine secretion [3,7–9], although some aspects of NOD signaling are thought to take place independently of NF-κB activation [10]. Notably, Nod2 knockout in mice impaired responses to MDP [11–13] and, conversely, expression

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of NOD2 can increase MDP signaling [5,14]. In addition, mutant forms of human NOD2 are associated with human inflammatory disorders [15–17].

Although MDP appears to require NOD2 for full activity, there is controversy concerning the primary receptor for MDP binding. It was first reported that NOD2 lacks any detectable affinity for immobilized MDP [18], and calretinin was suggested as a receptor target. A subsequent report described binding of purified NOD2 protein to biotinylated MDP, but excess biotinylated MDP retained only ~3% of NOD2 protein [19]. More recently direct binding of MDP to partially purified recombinant NOD2 protein produced in insect cells was described [20]. It remains an open question whether muramyl peptides bind exclusively to NOD2 or if other targets including calretinin contribute to the immunomodulatory activity of MDP. We therefore addressed the possibility that muramyl peptides might target an alternative receptor.

In earlier work we used monoclonal antibody against GMDP to probe a phase display library; this detected a GMDP mimetic peptidomimetic denoted ‘RN-15’ (RVPVRHYAKISPVMV) that, similarly to GMDP/MDP, augments antibody and cytokine responses in vivo following immunization, although it differs from GMDP in that it lacks pyrogenic activity [21]. More recently we reported expression cloning of GMDP-binding polypeptides from mouse splenomyelocytes; all clones identified multifunctional factor YB-1 as a specific target for GMDP [22].

In the present report we extend these preliminary findings. We now address specific GMDP binding to YB-1 and demonstrate that such binding leads to functional activation and induction of chemokine gene expression.

2. Methods

Regents used in this work including mouse monoclonal antibody to YB-1 were described previously [22]; further experimental details can be found in the Supplementary Methods online. Recombinant YB-1 was expressed in Escherichia coli, and purified as described previously [22,43,44].

Experiments use both WEHI-3 and bone marrow–derived dendritic cells. WEHI cells were grown as described (Supplementary Methods). Bone-marrow cells were prepared and subjected to dendritic maturation using recombinant GM-CSF. For knockdown of YB1 the antisense oligonucleotide was 5'-GGG GCC TCC TCC ACC CAA TTA C-3'; the control oligonucleotide of the same G+C content was 5'-TTG ATC TTC CCA TGG A-3'; these were stably expressed under a PolIII promoter following transfection and drug selection.

Nfkb2 transcript levels were measured by qPCR using primers 5'-ATG CCT GTT TAT GAG GGA TAT ACC CTA ACC-3' and 5'-GAC TTG ATC GTG CAG GCC TTA TCC-3' located within the downstream coding region of Nfkb2, and which identifies all major splice variants. Gapdh internal control primers were 5'-GAC CAC AGT CCA TGT GCG ACT CTG A-3' and 5'-GAC CTT GGT CAA CCA TCA GAT T-3' which were used as a control for gene expression.

For GMDP binding, primary mouse splenocytes were incubated with FITC-GMDP in the presence or absence of unlabeled GMDP (concentrations as specified in figure legends). After 2 h at 37 °C and washing cell lysates were prepared as before. Lysates were incubated with equal concentrations (as given previously) of biotinylated antibodies against YB-1 (purified mouse monoclonal, IgG) or with a control nonspecific antibody (purified total lg from immunized mice, predominantly IgG). Antibody complexes were collected by binding to streptavidin–magnetic beads as before. FITC fluorescence was measured spectrophuorometrically (excitation, 494 nm; emission 520 nm) on a Lumina fluorescence spectrometer.

For confocal microscopy, cells were activated with GMDP or FITC-GMDP for 4 h; nuclear stain Hoechst 33342 was added for the last hour of incubation. Cells were fixed/permeabilized (2% paraformaldehyde, 0.1% Triton X-100), washed, and stained with mouse antibody to YB-1 and developed with secondary anti-mouse IgG-AlexaFluor 555. Images were collected using an Eclipse E2000 confocal microscope (Nikon, Japan) equipped with 405, 488, and 514 nm lasers.

To analyze activation of NF-κB cleavage and nuclear translocation, primary mouse splenocytes were incubated with different combinations of GMDP and YB-1 for 4 h. Cells were lyzed, separated into nuclear and cytoplasmic fractions by centrifugation (10 min, 3000 g, 4 °C), and fractions analyzed by western blotting using a specific mouse antibody to NF-κB2 (Sc-298, Santa Cruz Biotechnology) that reacts with both the p100 precursor and the p52 activated cleavage product. After washing, the blot was developed using conjugated rabbit anti-mouse antibody as before and chemiluminescent substrate (Pierce ECL western blotting substrate 32106).

3. Results and discussion

We addressed direct binding of GMDP to YB-1 using fluorescent (FITC, fluorescein isothiocyanate)-labeled GMDP. Following incubation of primary mouse splenocytes with FITC-GMDP, extracts were immunoprecipitated with monoclonal antibody against YB-1; the complexes were disrupted and supernatants were examined for released FITC fluorescence. As shown in Fig. 1A, primary antibody against YB-1 retained FITC fluorescence (520 nm), whereas control antibody did not, demonstrating that YB-1 is present in a complex with FITC-GMDP. The fluorescence signal was abolished by excess unlabeled GMDP, demonstrating the specificity of the interaction.

We previously reported, using recombinant YB-1, that GMDP competes with FITC-GMDP for binding to YB-1 [22]. To estimate the extent of the association, we incubated a fixed concentration of FITC-GMDP with increasing amounts of YB-1 and examined retention of fluorescence by anti-YB-1 antibody. As shown in Fig. 1B, fluorescence retention was proportional to input YB-1, with saturation at approximately isomolar concentrations of YB-1 and FITC-GMDP, indicative of a 1:1 complex between the two molecules.

To provide further evidence for GMDP binding to YB-1 we examined their subcellular distributions in cells treated with labeled GMDP. Confocal fluorescence microscopy was performed on mouse bone marrow–derived dendritic cells using FITC-GMDP (yellow-green) in conjunction with fluorescent antibody (red) against YB-1. As shown in Fig. 2, GMDP colocalizes with YB-1 within predominantly cytoplasmic complexes. These observations are consistent with GMDP/YB-1 complex formation.

To address whether GMDP binding to YB-1 is functionally active, we examined the expression of nuclear factor κB (NF-κB2), a key mediator of the induction of innate immunity, in response to GMDP and YB-1. In a first experiment, the mouse monocyte WEHI-3 cell line was incubated with GMDP and/or recombinant YB-1. As shown in Fig. 3A, control cells showed marked upregulation by both GMDP and YB-1, with maximal upregulation in the presence of both added factors.

Because these cells endogenously express YB-1, and GMDP alone brought significant upregulation of Nfkb2 gene expression (Fig. 3A, left), we performed YB-1 knockdown of YB-1. Stable transformants of mouse monocyte cell line WEHI-3 expressing YB-1 or control antisense siRNA of the same G+C content were analyzed for Nfkb2 expression in response to GMDP. Knockdown robustly reduced both YB-1 protein and mRNA levels (Supplementary Methods)
Fig. S1). In cells knocked down for YB-1 the upregulation of \( \text{Nfkb2} \) by GMDP was largely abolished (Fig. 3A, right) whereas the control antisense had no effect (Supplementary Fig. S2). Importantly, GMDP-induced upregulation of \( \text{Nfkb2} \) was restored in YB-1 knock-down cells by exogenous recombinant YB-1 protein (1 \( \mu \)g/ml) (Fig. 3A, right). These results demonstrate that YB-1 is essential for induction of \( \text{Nfkb2} \) expression by GMDP.

MDP and GMDP contain a key L-Ala D-isoGln dipeptide that is required for biological activity. To confirm the specificity of the YB-1/GMDP interaction we used biologically inert LL-GMDP in which the L-Ala-D-isoGln grouping is replaced by inactive L-Ala-L-isoGln [6]. This was tested for induction of \( \text{Nfkb2} \) expression in a standard assay as before. As shown in Fig. 3B, LL-GMDP was inactive in stimulating \( \text{Nfkb2} \) induction. To exclude the possibility that contaminating bacterial LPS might contribute to the activity of recombinant YB-1 protein, the protein was pre-treated with trypsin; this abolished \( \text{Nfkb2} \) induction (Supplementary Fig. S3).

A key issue raised by this work was whether the expression of NF-\( \kappa B \) induced by GMDP and YB-1 is functional. To address this, we first examined whether NF-\( \kappa B \)2 protein is activated by GMDP/YB-1 stimulation. Following cell activation the NF-\( \kappa B \)2 precursor protein is cleaved to generate the active p52 fragment that is then translocated into the nucleus where it activates transcription of target genes [23]. We therefore examined NF-\( \kappa B \)2 cleavage and subcellular distribution following treatment with GMDP and/or YB-1. Accurate separation of nuclear and cytoplasmic was confirmed by western blotting for nuclear (CREB) and cytoplasmic (GAPDH) markers (Fig. S4).
As shown in Fig. 4, untreated cells contained little of either p100 or its cleavage product p52. Treatment with either GMDP or YB-1 led to a small increase in cytoplasmic p52 levels; however, the combination of both GMDP and YB-1 led to a significant increase in levels of both p100 and p52. The combination also promoted nuclear translocation of p52 (Fig. 4). Although some nuclear p52 was present in the absence of exogenous YB-1, which we ascribe to endogenous YB-1, again the combination of YB-1 and GMDP led to the greatest increase in nuclear levels of p52 (Fig. 4). We conclude that GMDP, with YB1, leads to activation and nuclear transport of NF-kB2.

To confirm that NF-kB2 activation and nuclear transport are functional in transcriptional activation, we next examined the levels of mRNA for a known NF-kB2-activated gene, Cxcr4, that encodes the chemokine (C-X-C motif) receptor 4, also known as fusin or LESTR. Cxcr4 is widely expressed in hematopoietic cells including lymphocytes, monocytes, macrophages, neutrophils, and eosinophils, and plays a key role in virus infection, innate immunity, and cancer [24,25]. Specifically, Cxcr4 transcription is known to be induced via NF-kB2-dependent pathways [26–28].

As shown in Fig. 4, expression of Cxcr4 was prominently upregulated by GMDP and YB-1. Although some induction was obtained with GMDP alone, which we ascribe to endogenous YB-1 expression (see Fig. 3A), maximal induction of Cxcr4 was with the combination of GMDP plus YB-1, confirming functional activation of NF-kB2-dependent transcription.

In summary, we suggested previously that GMDP may bind to multifunctional factor YB-1 [22], this is confirmed by direct binding assays in the present work. Because we have shown that GMDP/YB-1 induces Nfkb2 gene expression, followed by cleavage and nuclear translocation of NF-kB2 protein, accompanied by upregulation of the expression of a known NF-kB2-dependent gene, Cxcr4, we conclude that NF-kB2 induced by GMDP/YB-1 is functionally active. These findings demonstrate that cell stimulation with GMDP/YB-1 leads to cellular reprogramming and upregulation of innate immune pathway signaling. We conclude that binding to YB-1 contributes to the adjuvant and immunostimulatory activity of bacterial muramyl peptides.

YB-1 is a multifunctional transcription factor implicated in innate immunity by modulating the expression of multiple cytokines, chemokines, and their receptors, and also binds to RNA where it regulates mRNA stability and translation [29,30]. The importance of YB-1 is underlined by the finding that mice genetically deficient for YB-1 die in utero [31]. YB-1 is also secreted from the cell where it binds to cell surface Notch3 receptors [32], and there is evidence that YB-1 expression is induced in response to bacterial infection; for example, extracellular YB-1 is found in the serum of sepsis patients but not in healthy controls [33].

The finding that GMDP targets YB-1 adds a further dimension to the biological activity of GMDP because NOD2 has been regarded as a primary binding target for muramyl peptides. However, the evidence is not entirely consistent [18–20]. Furthermore, in macrophages, MDP can induce caspase-1 activation and IL-1 induction by a pathway independent of NOD2 [34], and the mitogenic and adjuvant activities of MDP can be separated [35]; these findings indicate that muramyl peptides have more than one target. Overall, the evidence presented here argues that multifunctional factor YB-1 provides a binding target for the biological action of the muramyl peptide GMDP. Further experiments will be necessary to address potential interactions between NOD2 and YB-1 signaling.

An important issue concerns the domain of interaction between YB-1 and GMDP. YB-1 is a highly conserved nucleic acid-binding protein with multiple biological functions in transcription and translation [29,36,37]. Although generally thought of as a cytosolic protein, YB-1 applied extracellularly is taken up into the cell (Fig. 5) and retains biological activity [32,38], as confirmed in the present report. Conversely, approximately 25% of YB-1 is secreted into the medium following LPS stimulation of monocytes [38]. It has been argued that YB-1 export follows a non-canonical pathway shared by IL-1β, macrophage inhibitory factor (MIF), and transcription factor HMGBl [32,38]. YB-1 thus appears to be a member of a specialized class of proteins that can cycle between the nucleus, cytosol, membrane, and the extracellular milieu, and as such are candidates for involvement in aspects of cell–cell paracrine signaling.
Notch family protein, Notch1, in macrophages [39]. Interestingly, NOD2 has been implicated in activation of another but this remains to be addressed.

exclude the possibility that, in intact cells, YB-1 and GMDP are part of YB-1/GMDP complexes. YB-1 can act as a binding partner for dipeptides will assist efforts to develop small-molecule therapeutics that can modulate inappropriate or insufficient immunological responses, and which would therefore have potential wide applicability to YB-1/GMDP complexes, and RNA-binding activity [40–42]; further experiments will be necessary to investigate this possibility.

Finally, the YB-1 system could also contribute to the induction of innate immunity by viral sRNA because YB-1 has both DNA- and RNA-binding activity [40–42]; further experiments will be necessary to investigate this possibility.

Conflict of interest


Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2015.05.028.

References


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