

Activation of a Rel-A/CEBP- β -Related Transcription Factor Heteromer by PGG-Glucan in a Murine Monocytic Cell Line

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Abstract PGG-Glucan is a soluble β -glucan immunomodulator that enhances a variety of leukocyte microbicidal activities without activating inflammatory cytokines. Although several different cell surface receptors for soluble (and particulate) β -glucans have been described, the signal transduction pathway(s) used by these soluble ligands have not been elucidated. Previously we reported that PGG-Glucan treatment of mouse BMC2.3 macrophage cells activates a nuclear factor κ -B-like (NF- κ B) transcription factor complex containing subunit p65 (rel-A) attached to an unidentified cohort. In this study, we identify the cohort to be a non-rel family member: a CCAAT enhancer-binding protein- β (C/EBP- β)-related molecule with an apparent size of 48 kDa, which is a different protein than the previously identified C/EBP- β p34 also present in these cells. C/EBP- β is a member of the bZIP family whose members have previously been shown to interact with rel family members. This rel/bZIP heteromer complex activated by PGG-Glucan is different from the p65/p50 rel/rel complex induced in these cells by lipopolysaccharide (LPS). Thus, our data demonstrate that PGG-Glucan uses signal transduction pathways different from those used by LPS, which activates leukocyte microbicidal activities and inflammatory cytokines. We further show that heteromer activation appears to use protein kinase C (PKC) and protein tyrosine kinase (PTK) pathways, but not mitogen-activated protein kinase p38. Inhibitor κ -B- α (κ B- α) is associated with the heteromer; this association decreases after PGG-Glucan treatment. These data are consistent with a model whereby treatment of BMC2.3 cells with PGG-Glucan activates κ B- α via PKC and/or PTK pathways, permitting translocation of the rel-A/CEBP- β heteromer complex to the nucleus and increases its DNA-binding affinity. *J. Cell. Biochem.* 77:221–233, 2000. © 2000 Wiley-Liss, Inc.

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PGG-Glucan is a soluble β -(1,6)-branched, β -(1,3)-linked glucose homopolymer isolated from the cell wall of *Saccharomyces cerevisiae* that exhibits microbial anti-infective activities unrelated to leukocyte cytokine induction [for review, see Bleicher and Mackin, 1995]. In animal models, PGG-Glucan enhances the clearance of intraperitoneal and intramuscular challenges of *Staphylococcus aureus* and *S. epidermidis* [On-

derdonk et al., 1992; Kernodle et al., 1998; Liang et al., 1998] and inhibits infection-stimulated alveolar bone loss [Stashenko et al., 1995]. The mechanism(s) of enhancement of these immunomodulatory microbicidal activities are currently under investigation but have been shown to include an increase in monocytes and neutrophils [Liang et al., 1998; Patchen et al., 1998a,b], as well as a potentiation of neutrophil oxidative burst activity [Liang et al., 1998]. Because PGG-Glucan does not induce proinflammatory cytokines [Poutsika et al., 1993; Adams et al., 1997; Liang et al., 1998; Wakshull et al., 1999], its mechanism of action may be different from the cytokine activation models proposed for lipopolysaccharide (LPS), derivatized-soluble β -glucans, or particulate β -glucans.

Receptors have been identified on the surface of leukocytes for soluble β -glucans [Konopski et

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al., 1994; Muller et al., 1996; Thornton et al., 1996], particulate β -glucans [Czop and Austen, 1985; Goldman, 1988; Czop and Kay, 1991; Szabo et al., 1995], and PGG-Glucan [Michalek et al., 1998; Zimmerman et al., 1998; Wakshull et al., 1999]. Treatment of leukocytes with particulate β -glucans has been shown to activate signal transduction pathways (presumably mediated by receptors) that involve protein kinase C (PKC) [Twomey et al., 1991; Qiu and Leslie, 1994; Tapper and Sundler, 1995; Harler et al., 1999], phospholipase A₂ (PLA₂) [Qiu and Leslie, 1994; Turner and Wood, 1994], PTK [Glaser et al., 1993; Fukushima et al., 1996], MAP kinase [Qiu and Leslie, 1994] and pertussis toxin-sensitive pathways [Lyman et al., 1988; Hiemstra et al., 1992]. However, the signal transduction pathways used by soluble β -glucans, including PGG-Glucan, are poorly understood.

Previously we demonstrated that treatment of mouse BMC2.3 macrophage cells with PGG-Glucan activates an NF- κ B-like transcription factor complex containing subunit p65 (rel-A) attached to an unidentified cohort with a size of approximately 50 kDa [Adams et al., 1997]. In these cells, LPS activated classic NF- κ B (p65/p50), increased the nuclear titer of p65 and p50 antigens, and increased cytokine (interleukin-1 β [IL-1 β], tumor necrosis factor- α [TNF- α]) mRNA production. By contrast, PGG-Glucan did not induce classic NF- κ B, it increased the nuclear titer of p65, but not of p50, and did not induce cytokine mRNA production. These data demonstrated that PGG-Glucan activated different transcription factors than LPS, and suggested that the PGG-Glucan-stimulated factors were not sufficient for increasing cytokine mRNA transcription.

In this report, we identify the previously unknown cohort in the PGG-Glucan-activated NF- κ B-like complex to be a new C/EBP- β -related protein, a member of the non-rel family bZIP. We also extend our knowledge of the PGG-Glucan activation of these cells to implicate the involvement of I κ B- α , PKC, and PTK.

MATERIALS AND METHODS

Materials

PGG-Glucan (Betafectin) manufactured by Alpha-Beta Technology (Worcester, MA) was determined to be endotoxin-free by a *Limulus* amoebocyte lysate assay (LAL assay; QCL-1000; Whittaker Bioproducts). *Escherichia coli* LPS was

purchased from Sigma Chemical Company (St. Louis, MO). Protease inhibitors (phenylmethylsulfonyl fluoride [PMSF], chymostatin, pepstatin A, aprotinin, antipain, and leupeptin) and other routine chemicals were purchased from Sigma or Boehringer-Mannheim (Indianapolis, IN). Calphostin-C (CAL), Genistein (GEN; 4',5,7-trihydroxyisoflavone), and MG-132 (MG; carbobenzoxy-leuciny-leuciny-leucinal) were purchased from Calbiochem. DNA oligomers were custom synthesized (Genosys Biotechnologies, Woodlands, TX). Antibodies to transcription factor proteins were purchased from Santa Cruz Biotechnology, except for C/EBP- β (GENEKA 4500786). All are polyclonal IgG induced against synthetic peptides with cross-reactivity for mouse: p65 (rel-A, rabbit anti-human, SC-372X), C/EBP- α (p42, rabbit anti-rat, SC-61X), C/EBP- δ (NF-IL-6 β , rabbit anti-rat, SC-151X), I κ B- α (rabbit anti-human, SC-371).

Cell Stimulations

Murine bone marrow-derived macrophage cells (BMC2.3, kindly provided by K.L. Rock, UMMS, Worcester, MA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 1 mM glutamine, and 1% Penn/Strep (all from Life Technologies, Gaithersburg, MD). LPS or PGG-Glucan were added to the media at final concentrations of 1 μ g/ml and 3 μ g/ml, respectively, and 37°C incubations continued for 15–60 min before preparation of nuclear extracts. During inhibitor experiments, Calphostin C (250 nM), Genistein (15 μ M), or SB-203580 (3 μ M) were added to the medium 5 min before stimulation.

Nuclear Extractions for Electrophoretic Mobility Shift Assays

Nuclear extracts for electrophoretic mobility shift assays (EMSA) were prepared as described previously [Adams et al., 1997], using 1.0–2.0 $\times 10^7$ cells per sample. All buffers were freshly supplemented with (final concentrations indicated in parentheses): DTT (0.5 mM), protease inhibitors PMSF (0.5 mM), chymostatin, pepstatin A, aprotinin, antipain, and leupeptin (each at 1 μ g/ml), and phosphatase inhibitors NaF (10 mM), ZnCl₂ (1 mM), sodium orthovanadate (1 mM), and sodium pyrophosphate (5 mM). Aliquots of the final dialysates were stored at –80°C and discarded after use.

Protein concentrations varied within 0.5–2.0 $\mu\text{g}/\mu\text{l}$, as determined by a modified Coomassie protein assay (Pierce) against a bovine serum albumin (BSA) standard.

Nuclear Extractions for Western Blots

Nuclear extracts for Western blots were prepared as previously described [Adams et al., 1997]. Cells ($1.0\text{--}2.0 \times 10^7$) were harvested at 4°C into PBS, then resuspended in 5 ml of hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 1.5 mM MgCl_2) freshly supplemented with DTT and the protease/phosphatase inhibitors described above. Cells were incubated 15 min on ice to permit swelling and penetration of protease inhibitors, then lysed by the addition of Nonidet P-40 (NP-40) to 0.5% (v/v) and gentle inversion. Nuclei were pelleted (1,500g, 5 min, 4°C) and resuspended in 250 μl of extraction buffer (20 mM Hepes pH 7.9, 0.45 M NaCl, 1 mM EDTA) freshly supplemented with inhibitors as above. Nuclear suspensions were incubated for at least 30 min at 4°C with intermittent agitation, then centrifuged (14,000g, 5 min, 4°C) to pellet nuclear debris. Supernatants were mixed with an equal volume of dilution buffer (20 mM Hepes pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 20% glycerol). Aliquots were stored at -80°C and discarded after use. Protein concentrations varied within 1–4 $\mu\text{g}/\mu\text{l}$.

Whole Cell Extracts for Western Blots

Whole cell extracts were used for $\text{I}\kappa\text{B-}\alpha$ immunoblots. BMC2.3 cells ($1.0\text{--}2.0 \times 10^7$ per sample) were washed in $1\times$ phosphate-buffered saline (PBS), 20 mM EDTA, then resuspended in 250 μl of fresh lysis buffer (20 mM Hepes pH 7.9, 10 mM KCl, 300 mM NaCl, 1 mM MgCl_2 , 0.1% Triton-X 100, 20% glycerol, 0.5 mM DTT, freshly supplemented with inhibitors as described above. Suspensions were incubated for at least 10 min on ice to lyse the cells, then centrifuged (14,000g, 5 min, 4°C) to pellet cell debris. Supernatant aliquots were stored at -80°C and discarded after use. Protein concentrations varied within 2–6 $\mu\text{g}/\mu\text{l}$.

Electrophoretic Mobility Shift Assays

Transcription factor activations were assayed using an EMSA as we described previously [Adams et al., 1997]. NF- κB consensus synthetic duplex probe [Lenardo and Baltimore, 1989] was 5'-AGTTGAGGGGACTTT-

CCCAGGC. Duplex probe was end-labeled with ^{32}P , using polynucleotide kinase and [$\gamma\text{-}^{32}\text{P}$]ATP. Labeled probe (0.5 pmol) was mixed with 3 μg of nuclear extract protein in a solution containing 10 mM Tris-HCl pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 5% glycerol, 0.02% β -mercaptoethanol, 0.1–1.0 μg of poly(dI/dC) (Pharmacia). Reactions were incubated at 25°C for 20 min to permit complex formation, then electrophoresed under nondenaturing conditions through 4% polyacrylamide gels in $0.5\times$ TBE buffer (45 mM Tris base, 45 mM boric acid, 1 mM EDTA). Gels were dried onto 3MM paper. Bands were visualized by autoradiography at -80°C with one intensifying screen, and quantitated by laser densitometry.

EMSA Probe Competitions

Nonradiolabeled "cold" synthetic duplex oligomers NF- κB (see above), NF-IL-6 [Mahoney et al., 1992] (5'-TGCAGATTGCGC-AATCTGCA), mutant NF- κB [Lenardo and Baltimore, 1989] (5'-AGTTGAGGCGACTTT-CCCAGGC), or mutant NF-IL-6 [Mahoney et al., 1992] (5'-TGCAGAGACTAGTCTCTGCA) were added to the EMSA reaction for 20 min at 25°C before ^{32}P -probe was added. After the addition of radiolabeled probe, the incubation was continued at 25°C for an additional 20 min before electrophoresis.

EMSA Antibody Competitions

Antibody to a specific transcription factor protein (see under Materials and Methods) (3 μg) was added to a 4°C EMSA reaction for 60 min before ^{32}P -probe was added. After labeled probe was added, incubations were continued at 25°C for an additional 20 min before electrophoresis.

Western Blots

Western blots were performed as described previously [Adams et al., 1997], using 5 μg of nuclear extract protein per lane for p65, 10 μg for p48, and 20 μg for $\text{I}\kappa\text{B-}\alpha$. Primary antibody incubations were for 2 h at 25°C in fresh blocking buffer ($1\times$ PBS, 1% casein, and 0.2% Tween-20) containing rabbit anti-human p65 (SC-372X, 0.1 $\mu\text{g}/\text{ml}$ final concentration), rabbit anti-rat p48 (GENEKA 4500786, 0.2 $\mu\text{g}/\text{ml}$ final), or rabbit anti-human p38 $\text{I}\kappa\text{B-}\alpha$ (SC-371, 0.2 $\mu\text{g}/\text{ml}$ final concentration, 1:1,000). Secondary antibody incubations were for 2 h at 25°C in fresh blocking buffer containing goat anti-

rabbit-HRP IgG (Pierce 31460, 0.4 $\mu\text{g/ml}$). Detection of biotinylated marker proteins (Bio-Rad, Broad Range) used streptavidin-horseradish peroxidase (HRP) (Pierce 21126, 0.5 $\mu\text{g/ml}$). Enhanced chemiluminescence (ECL) detection of HRP used luminol/ H_2O_2 (Pierce). Exposure for autoradiography was at 25°C for 1–5 min.

Preparative Immunoprecipitations

Anti-C/EBP- β or anti-p65 (see under Materials and Methods) (100 μg) were linked to 500 μl of Carbolink™ gel (Pierce) via the oxidized carbohydrate IgG moiety, using the manufacturer's procedure. Approximately 80% of the IgG coupled to the resin (0.16 μg IgG/ μl resin). Resin (20 μl of 50% slurry in PBS, 1.6 μg IgG) was added directly to nuclear extracts (200 μg), and the samples were incubated overnight at 4°C with constant shaking. Beads were washed 3 \times in PBS, 0.05% Triton X-100; the proteins were eluted in protein electrophoresis sample buffer by boiling for 2 min. Samples were analyzed on immunoblots, using p65, C/EBP- β , or $\text{I}\kappa\text{B}-\alpha$ antibodies (see Western blot protocol).

RESULTS

NF- κB Activated by PGG-Glucan Is Not Classic NF- κB

In our previous study, PGG-Glucan was found to activate an NF- κB -like complex that strongly reacted with rel-A antibody, but not with $\kappa\text{B}1$, $\kappa\text{B}2$, rel-B, or C-rel antibodies [Adams et al., 1997]. The size of this rel-A protein in BMC2.3 cells was shown to be 65 kDa (similar to other mammalian cells), yet the size of the EMSA complex was inconsistent with its containing a p65/p65 homodimer. Thus, we hypothesized that the complex may contain a second non-rel subunit. Studies in other laboratories demonstrated that rel family members (e.g., p65) are capable of interacting with members of other transcription factor families, especially the bZIP family (which includes NF-IL-6) [Stein et al., 1993; Diehl and Hannink, 1994; Ray et al., 1995]. We tested the ability of an NF-IL-6 consensus probe (representing bZIP) to compete with ^{32}P -labeled NF- κB consensus probe (previously shown to bind our NF- κB -like complex) for complex formation. LPS-treated BMC2.3 cells were used as a positive control, since treatment of macrophage cells with this agent is well known to activate

both classic NF- κB (a p65/p50 rel/rel complex) and NF-IL-6 (a C/EBP- β -homodimer complex) [for review, see Baldwin, 1996; Sweet and Hume, 1996; Barnes and Karin, 1997].

One predominant DNA/protein complex formed (Fig. 1, middle of A, B, and C), as we previously observed, using optimal stimulation with either LPS (1 $\mu\text{g/ml}$) or PGG-Glucan (3 $\mu\text{g/ml}$) for 60 min [Adams et al., 1997]. For LPS-treated cells (Fig. 1A), cold NF-IL-6 consensus probe did not compete for NF- κB complex formation (even at 50-fold molar excess relative to labeled probe) as expected for classic NF- κB . However, for PGG-Glucan-treated cells, cold NF-IL-6 probe strongly competed for NF- κB complex formation (Fig. 1B), while cold mutant NF-IL-6 probe did not (Fig. 1C). These data indicate that, in PGG-Glucan-treated cells, the predominant complex bound to the NF- κB consensus probe is not classic NF- κB ; in addition, it may contain a member of the NF-IL-6 family of proteins.

NF- κB Activated by PGG-Glucan Contains a C/EBP- β -Related Component

Antibody-supershift EMSA was performed (Fig. 2) to determine the specific protein subunits of the predominant EMSA complex. All antibodies used for these experiments had cross-reactivity for the appropriate mouse protein (see under Materials and Methods). C/EBP- γ antibody was not tested because it was not commercially available. For the LPS-stimulated extracts (Fig. 2A), neither preimmune serum nor any of the NF-IL-6 antibodies tested (C/EBP- α , β , δ) competed for NF- κB complex formation, as expected for a complex containing only rel components. By contrast, for PGG-Glucan-stimulated extracts (Fig. 2B), anti-C/EBP- β completely supershifted the predominant complex to a higher position (marked with an asterisk). Combined with our previous rel-A data, these data indicate that the EMSA complex most likely contains antigens related to rel-A and C/EBP- β , and is an interfamily "heteromer."

In order to determine whether rel-A and C/EBP- β are indeed physically associated in PGG-Glucan-stimulated BMC2.3 cells, immunoprecipitation experiments were performed (Fig. 3). Nuclear extracts were prepared from cells stimulated with LPS or PGG-Glucan and then incubated with Carbolink™ beads attached to C/EBP- β antibody (Fig. 3A) or beads

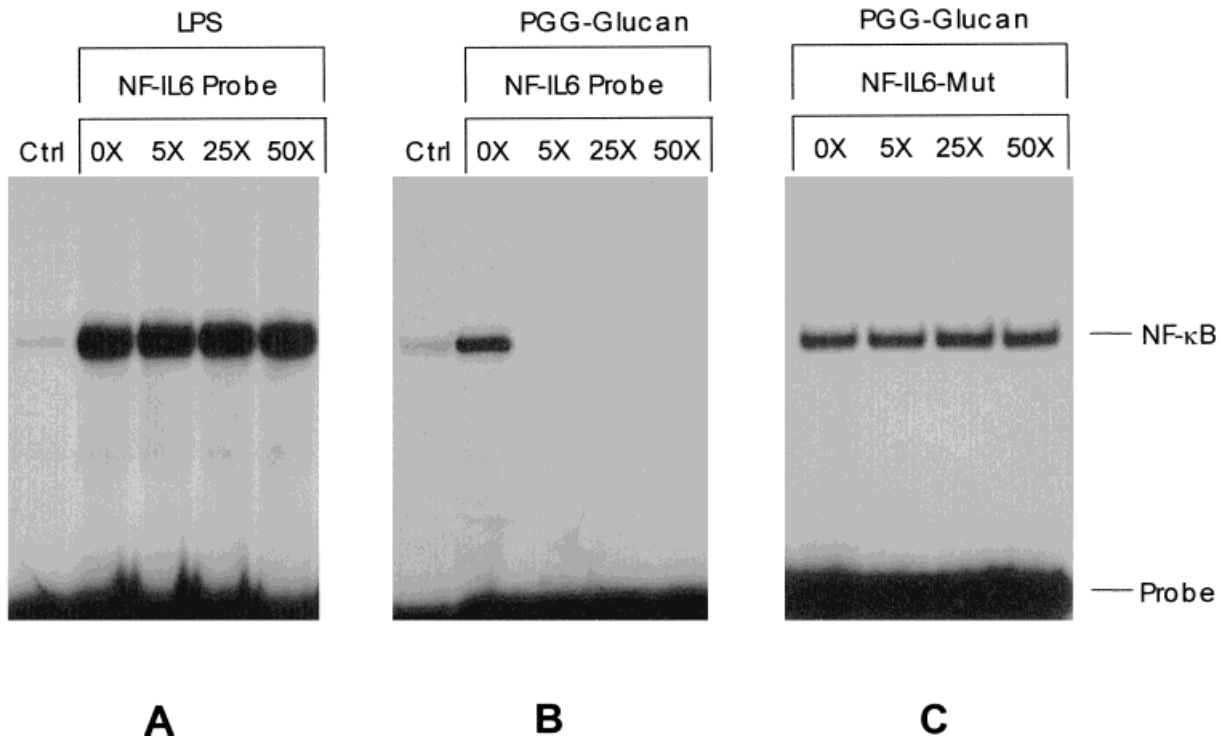


Fig. 1. Electrophoretic mobility shift assay (EMSA) cold probe competition. EMSA was performed using NF- κ B consensus probe. Various molar excess quantities (amounts are indicated relative to the 0.5-pmol of radiolabeled probe added to each reaction) of nonradiolabeled "cold" oligomer or "cold" mutant oligomer (see under Materials and Methods) were added to the

lipopolysaccharide (LPS)- (1 μ g/ml, 60 min) (A) or PGG-Glucan-stimulated (3 μ g/ml, 60 min) (B,C) BMC2.3 nuclear extracts before addition of 32 P-probe. Lanes designated 0x represent stimulated extracts not competed with cold probe. Upper horizontal line, position of the predominant EMSA complex; lower line, radiolabeled free probe.

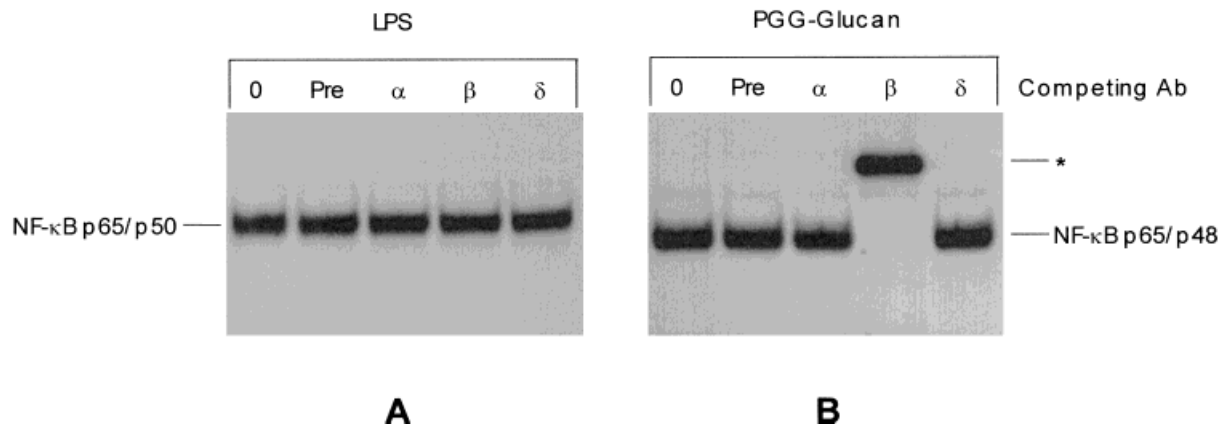


Fig. 2. Antibody supershift of the electrophoretic mobility shift assay (EMSA) complex. EMSA was performed using NF- κ B consensus probe as described under Materials and Methods. Competing antibody (3 μ g, type indicated above each lane) was added to a 3- μ g aliquot of LPS- (1 μ g/ml, 60 min) (A) or PGG-Glucan-stimulated (3 μ g/ml, 60 min) (B) nuclear extract before

32 P-probe addition. The following are antibody designations (see under Materials and Methods): 0 (no competing antibody), pre (preimmune), α (C/EBP- α), β (C/EBP- β), δ (C/EBP- δ). Upper horizontal line marked with an asterisk denotes the position of supershifted complex.

attached to rel-A antibody (Fig. 3B). Proteins eluted from the C/EBP- β -beads were analyzed by rel-A immunoblots, while those eluted from

the rel-A beads were analyzed by C/EBP- β immunoblots. For untreated extracts (Ctrl), or those treated with LPS, no rel-A (p65) antigen

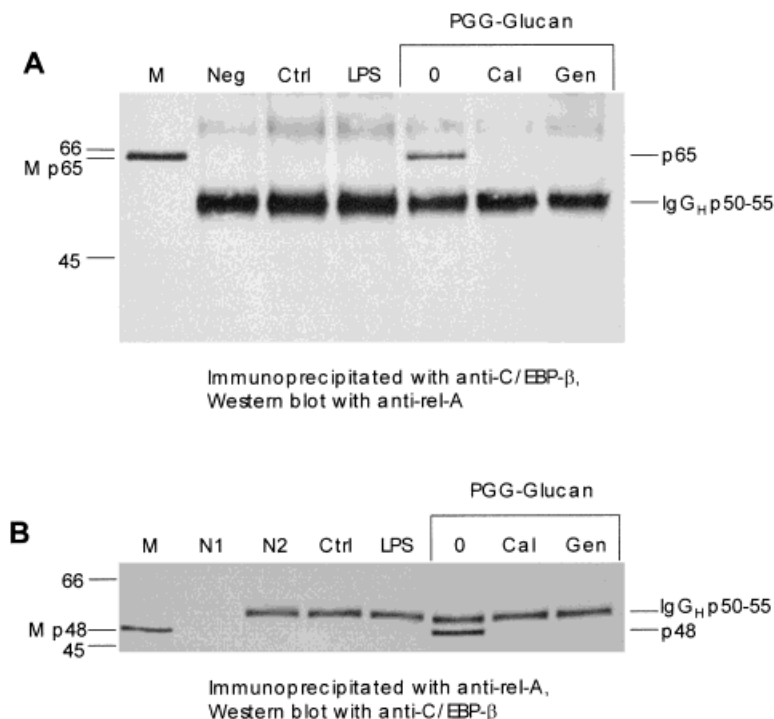


Fig. 3. Immunoprecipitation of p65/p48 heteromer. BMC2.3 nuclear extracts prepared from lipopolysaccharide (LPS)- (1 μ g/ml, 60 min) or PGG-Glucan-treated cells (3 μ g/ml, 60 min) were incubated with anti-C/EBP- β -beads (**A**) or anti-rel-A-beads (**B**), followed by analysis on an anti-p65 immunoblot (**A**) or anti-p48 immunoblot (**B**). **Lanes M**, nonimmunoprecipitated PGG-Glucan-treated nuclear extracts as antigen markers; **lanes Neg,N2**, IgG-Carbolink™ not mixed with nuclear extract to show the position of leached denatured IgG_H; **lane N1**, beads without antibody mixed with extract to verify lack of nonspecific binding; **lanes Ctrl**, IgG-beads mixed with unstimulated extract; **lanes Cal, Gen**, pretreated with these inhibitors (see under Materials and Methods) for 5 min before PGG-Glucan activator was added. Horizontal line to the right, position of p65 (rel-A) antigen, p48 (C/EBP- β -related) antigen, and p50–55 putative denatured IgG heavy-chain leached from the matrix (IgG_H). Horizontal line to the left, positions of biotinylated markers (sizes in kDa), or antigen markers.

was isolated by the C/EBP- β -immunoprecipitation. By contrast, C/EBP- β -immunoprecipitated extracts stimulated with PGG-Glucan (lane 0 in Fig. 3A) showed a clear p65 band, which co-migrated with the predominant rel-A antigen present in nonimmunoprecipitated whole cell extracts (lane M). This immunoprecipitated p65 signal did not result from nonspecific binding of nuclear proteins to the beads because unstimulated immunoprecipitated cell extracts (Ctrl) produced no signal. All treatments of the C/EBP- β -beads showed a strong band approximately 50–55 kDa in size that represents IgG heavy-chain denatured from the beads by our elution technique (boiling in β -mercaptoethanol-containing sample buffer) and detected by our anti-rabbit secondary antibody (lane Neg), but this strong band did not obscure the p65 antigen.

The rel-A-immunoprecipitation experiment (Fig. 3B) showed similar data, except in this case the immunoprecipitated extracts stimulated with PGG-Glucan (lane 0) showed a band of about 48 kDa that migrated just beneath the 50- to 55-kDa IgG_H and co-migrated with one of two predominant C/EBP- β antigens present in nonimmunoprecipitated whole cell extracts (Fig. 3, lane M, and Fig. 4). Previous experiments showed the size of C/EBP- β to be 40 kDa in

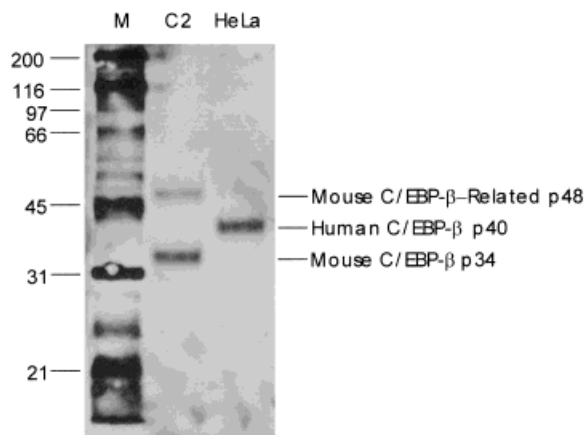


Fig. 4. Sizes of the C/EBP- β antigens in BMC2.3 cells. Whole cell extracts were prepared from BMC2.3 cells (**lane C2**) or HeLa cells (**lane H**), and 10 μ g of protein was analyzed by a C/EBP- β immunoblot. Horizontal lines to the right, positions of previously described C/EBP- β antigens (p34, p40) or the novel C/EBP- β -related antigen (p48). Horizontal lines to the left, positions of biotinylated markers (**lane M**) (sizes in kDa).

human HeLa cells [Williams et al., 1991] and 34 kDa in mouse liver [Williams et al., 1995]. The C/EBP- β antibody used in this study detected these previously characterized human and mouse C/EBP- β antigens in whole cell extracts (Fig. 4) but also detected a new weaker 48-kDa C/EBP- β -related antigen in the C2 cells, the

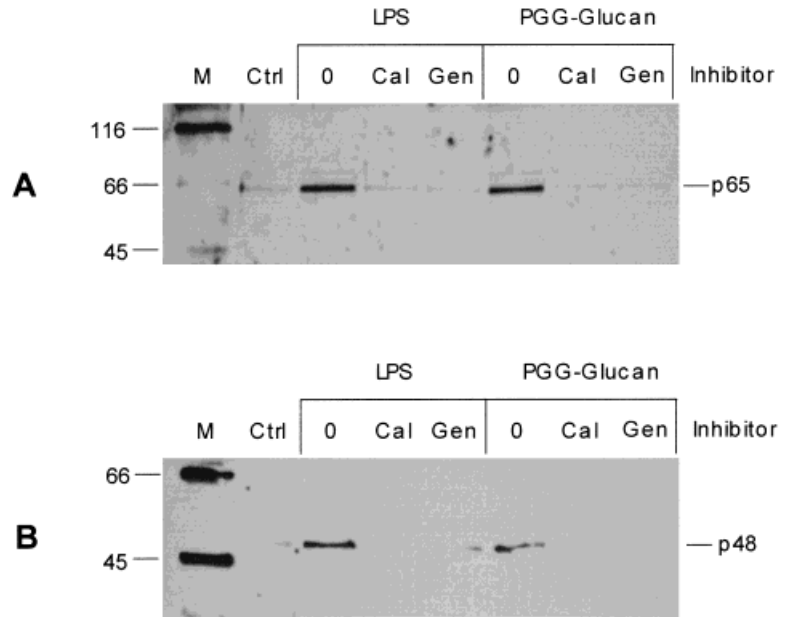


Fig. 5. Effects of inhibitors on nuclear titers of p65 and p48. BMC2.3 nuclear extracts prepared from lipopolysaccharide (LPS)- (1 $\mu\text{g/ml}$, 60 min) or PGG-Glucan-treated cells (3 $\mu\text{g/ml}$, 60 min) were analyzed by anti-rel-A (A) or anti-C/EBP- β (B) immunoblots. **Lanes Ctrl**, unstimulated extracts; **lanes 0**, uninhibited extracts treated with the indicated activator; **lanes Cal, Gen**, extracts prepared from cells pretreated with calphostin-C or genistein, respectively, for 5 min before activation. Horizontal lines to the right, antigens p65 or p48; horizontal lines to the left, positions of biotinylated markers (sizes in kDa).

same size as the heteromer-associated C/EBP- β antigen. The size of this 48-kDa antigen is also similar to that of the main C/EBP- β antigen translocated into the nucleus after treatment with LPS or PGG-Glucan in nonimmunoprecipitated extracts (Fig. 5). The size of this new C/EBP- β -related antigen is close to that of p50; thus, its size may help explain why the LPS-activated p65/p50 EMSA complex appears to have about the same mobility on nondenaturing gels as the PGG-Glucan-activated p65/p48 complex. In some experiments, the heteromer appears to migrate slightly faster than classic NF- κB (Figs. 1, 2), but in other experiments they appear to co-migrate (see Fig. 7). These immunoprecipitation data demonstrate that p65 (rel-A) and a 48-kDa protein immunologically related to, but distinct from, classic C/EBP- β appear to be attached together in BMC2.3 nuclei stimulated with PGG-Glucan, and the titer of this complex increases in the nucleus after PGG-Glucan treatment.

Potential Association of I κB - α With the Heteromer

In resting cells, an NF- κB factor (composed of two subunits) is usually present in the cytosol in an inactive state, complexed with an inhibitor κB - α (I κB - α) to make a trimer [for review, see Baldwin, 1996; Sweet and Hume, 1996; Barnes and Karin, 1997; Krappmann and Scheidereit, 1997; Parry and Mackman, 1998]. Activation of this trimer complex occurs through phosphoryla-

tion of I κB - α at serines 32 and 36. I κB dissociates from the complex, is ubiquitinated, and is subsequently degraded by a 26S proteasome complex. Removal of I κB from NF- κB exposes the nuclear localization signal on the NF- κB rel subunits, leading to their translocation to the nucleus. Because the heteromer contains a rel subunit, we hypothesized that the heteromer might also exist in trimer formation. Heteromer was purified from BMC2.3 whole cell extracts, using anti-C/EBP- β attached to CarbolinkTM beads; the bound material was analyzed by anti-I κB - α immunoblots (Fig. 6). All samples (except the one activated by PGG-Glucan) contained a strong band at 38 kDa, whose size matches that of I κB - α in a variety of other cells (see above reviews). LPS did not induce a change in C/EBP- β -associated I κB - α , however a complete disappearance of the bound inhibitor occurred after 15-min treatment with PGG-Glucan (lane 0). These data demonstrate an association of the inhibitor with C/EBP- β (and most likely heteromer) in these cells, which is quickly disrupted by PGG-Glucan.

Involvement of PKC and PTK

Inhibition experiments were performed to investigate which kinase pathways might be involved in activating the heteromer. Calphostin-C (CAL) is a cell-permeable highly specific inhibitor of the PKC family of proteins [for review, see Tamaoki and Nakano, 1990]. The specificity of CAL results from its interaction

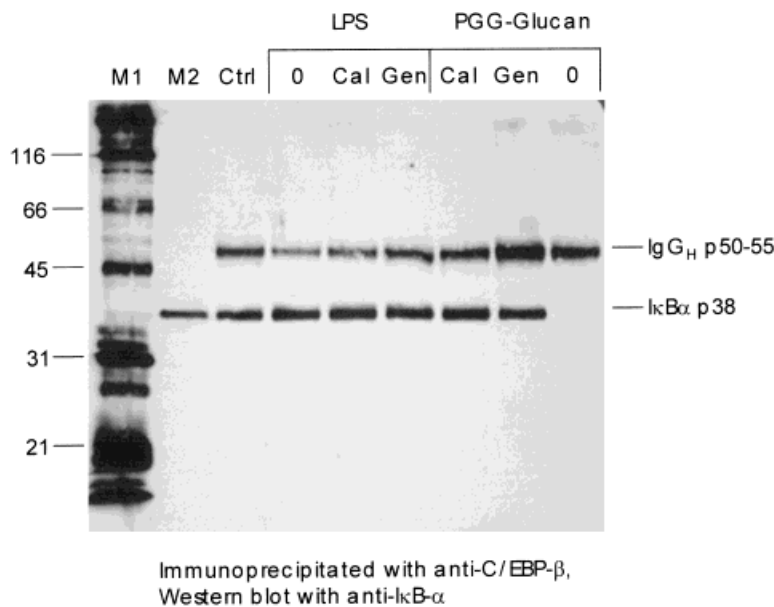


Fig. 6. Immunoprecipitation of I κ B bound to C/EBP- β . BMC2.3 whole cell extracts prepared from LPS- (1 μ g/ml, 15 min) or PGG-Glucan-treated cells (3 μ g/ml, 15 min) were incubated with anti-C/EBP- β -beads as described under Materials and Methods, followed by analysis on an anti-I κ B- α immunoblot. **Lane M1**, biotinylated marker; **lane M2**, nonimmunoprecipitated nonactivated whole cell extract marker for I κ B- α ; **lane Ctrl**, beads (coupled to antibody) mixed with unstimulated extract. The remaining lanes are as indicated in Fig. 5. Horizontal lines to the right, positions of 38 kDa I κ B- α antigen (lower) and IgG_H leached from the beads; horizontal lines to the left, positions of biotinylated markers (sizes in kDa).

with this family's unique regulatory domain, not the catalytic domain common to other kinases. Genistein (GEN) is a highly specific inhibitor of tyrosine kinases (IC₅₀ 2.6 μ M) with little effect for serine- and threonine-specific protein kinases, including PKC [Akiyama et al., 1987; Plataniias and Colamonici, 1992]. SB-203580 (SB) is a highly specific and cell-permeable inhibitor of p38 type MAPK (IC₅₀ 600 nM) [Lee et al., 1994; Cuenda et al., 1995]. Figure 7 shows the results of EMSA, using NF- κ B-consensus probe for BMC2.3 cells pretreated with CAL or GEN (Fig. 7A) or SB (Fig. 7B) at $5 \times$ IC₅₀ concentrations for 5 min before activation with LPS or PGG-Glucan. LPS was used for comparison in this investigation, as this stimulator is known to use a PKC- ζ (PKC- ζ) isoform to activate NF- κ B in macrophage cells [Lozano et al., 1994], and GEN has been shown to block the LPS-induced activation of NF- κ B (and increase in cytokine production) in human monocytes [Geng et al., 1993]. Treatment of BMC2.3 cells with any of the three inhibitors before LPS activation prevented formation of classic NF- κ B as expected from previous studies. CAL and GEN, but not SB, blocked activation of the PGG-Glucan-induced heteromer. None of the inhibitors was found to lower cell viability (viability remained >90%); thus, the blocked EMSA signals did not result from cell death. CAL and GEN were also found to block the PGG-Glucan-induced appearance of heteromer in the nucleus (Fig. 3),

the appearance of rel-A and C/EBP- β antigens in the nucleus (Fig. 5), and the dissociation of I κ B- α from C/EBP- β (Fig. 6). Altogether, these inhibition data demonstrate that activation of heteromer by PGG-Glucan appears to involve PKC and PTK, but not p38 MAPK. Because CAL and GEN block all three types of activation events monitored in this study (I κ B dissociation from C/EBP- β , heteromer translocation, and heteromer DNA affinity), these three events may be related within the PGG-Glucan-treated BMC2.3 cell.

DISCUSSION

This study demonstrates that PGG-Glucan-treatment of mouse BMC2.3 macrophage cells activates a rel-A/C/EBP- β -related transcription factor heteromer (Figs. 1–3) and increases its concentration in the nucleus (Fig. 3). The rel-A subunit of the heteromer was shown to have an apparent size of about 65 kDa (Fig. 3A), which matches that of the predominant rel-A antigen in these cells (Fig. 5A). The C/EBP- β -related subunit of the heteromer was shown to have an apparent size of about 48 kDa (Fig. 3B), which is close to the 50-kDa size previously predicted by us [Adams et al., 1997], matches that of a new C/EBP- β -related antigen in these cells (Fig. 4), and is the main one translocated into the nucleus after PGG-Glucan treatment (Fig. 5B). Because the previously unidentified subunit is not a rel family member, these data explain our earlier finding that the predomi-

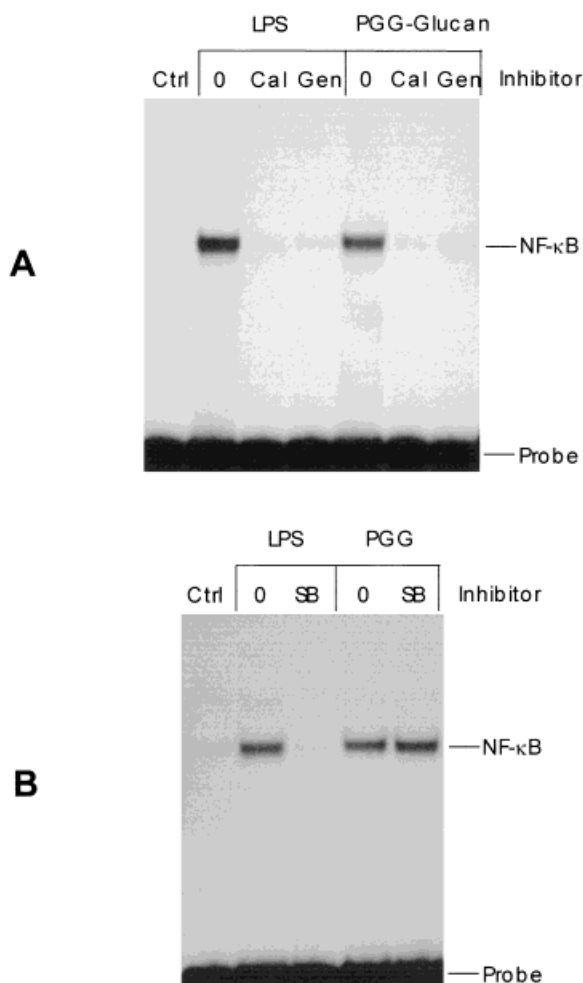


Fig. 7. Electrophoretic mobility shift assay (EMSA) analysis of inhibitor-treated extracts. Nuclear extracts were prepared from untreated cells (Ctrl), or cells treated for 60 min with lipopolysaccharide (LPS) (1 μ g/ml) or PGG-Glucan (3 μ g/ml) and analyzed by EMSA. Lanes as described in Fig. 5, except lane SB was pretreated with SB-203580 for 5 min before activation. Upper horizontal line, position of the predominate EMSA complex bound to the NF- κ B consensus probe; lower line, free probe.

nant complex bound to the NF- κ B consensus probe in PGG-Glucan-stimulated BMC2.3 cells did not interact with any rel antibody other than p65 [Adams et al., 1997]. These data also confirm our earlier conclusions that activation of an NF- κ B-like factor by PGG-Glucan does not result from potential trace amounts of LPS endotoxin contamination of our β -glucan preparation; otherwise, we would have detected the classic LPS-activated p65/p50 complex in PGG-Glucan-treated cells.

This new C/EBP- β -related antigen appears to be different from previously described C/EBP- β proteins, especially for mouse cells.

Previously characterized C/EBP- β proteins display a variety of apparent sizes depending on the cell source [for review, see Wedel and Ziegler-Heitbrock, 1995; Lekstrom-Himes and Xanthopoulos, 1998]. For example, C/EBP- β is 45 kDa in chicken myelomonocytic cells [Katz et al., 1993], 44 kDa in human liver cells [Natsuka et al., 1991], 40 kDa in transfected HeLa cells [Williams et al., 1991], and 34 kDa in mouse liver cells [Williams et al., 1995]. Even within one cell type, multiple C/EBP- β isoforms have been shown to exist, apparently resulting from AUG start site multiplicity. In mouse liver, these C/EBP- β isoforms have reported sizes of 42, 35, and 20 kDa [Sears and Sealy, 1994], and 35, 20, and 8–16 kDa [An et al., 1996; Hsieh et al., 1998]. Because our 48-kDa protein appears to be larger than any of these previously characterized C/EBP- β , it may be a newly characterized C/EBP- β isoform, or perhaps a novel protein immunologically related to C/EBP- β . Sequence analysis of the 48-kDa protein will aid identification.

To our knowledge, protein complexes containing rel-A and C/EBP- β have not previously been identified *in vivo*, although *in vitro* transfection studies have shown that all members of the rel and C/EBP families are theoretically capable of interacting [Stein et al., 1993]. A complex containing p66 (believed to be rel-A), C/EBP- δ , and at least three other unidentified proteins has been isolated from the avian T-cell line MSB-1 [Diehl and Hannink, 1994]. A complex containing p65 and C/EBP- δ has been isolated from the liver of LPS-treated rabbits; however, the composition of the complex is unknown, possibly containing a multimer of the heteromer [Ray et al., 1995]. In some cases, dimerization of these inter-family members occurs via direct interaction of the rel and leucine zipper domains present within the rel and bZIP components, respectively [LeClair et al., 1992; Stein et al., 1993].

We have not yet elucidated the function of this new heteromer. Specific genes activated by PGG-Glucan have yet to be identified (although our preliminary data suggest *c-Fos* is transcribed), so it is premature to analyze promoter sequences that may be activated/repressed by the heteromer. Based on the literature, it is also difficult to speculate on its function. In general, the DNA-binding domains and functions of these rel/bZIP heteromers remain unclear. It is unknown whether heteromers bind to separate rel and

bZIP DNA motifs within promoters bringing the two motifs into close proximity, whether they bind novel overlapping or adjacent rel/bZIP motifs, or whether only one subunit binds DNA. Functionally, rel/bZIP heteromers have been shown to inhibit promoters with κ B enhancer motifs [Stein et al., 1993]. This agrees with a previous finding that the interaction of both subunits of classic NF- κ B to a promoter is required for transcriptional activation [Kunsch et al., 1992]. Thus, the PGG-Glucan-activated heteromer (containing only one rel subunit) may physically bind κ B sites (including the EMSA probes used in the present study), but it may not contribute to the transcription of cytokine mRNAs from their κ B-containing promoters. In support of this, we have shown that LPS treatment of RAW264.7 macrophage cells (we are currently unable to transfect BMC2.3 cells) transfected with an NF- κ B-dependent reporter stimulates reporter transcription by fivefold, while stimulation of these cells with PGG-Glucan produces no detectable stimulation of the reporter (data not shown). If RAW macrophage cells contain a heteromer identical to BMC2.3 macrophage cells, these data show that the heteromer does not stimulate transcription from classic NF- κ B promoter sites. Thus, our previous finding that PGG-Glucan does not activate cytokine genes in BMC2.3 cells [Adams et al., 1997] may be explained in part by the PGG-Glucan-induced activation of p65/p48, which binds κ B motifs within cytokine promoters, blocking their transcription. However, this inhibition mechanism of action is not supported by our preliminary data (not shown) that cells co-incubated with LPS and PGG-Glucan still upregulate cytokines.

Other studies indicate that rel/bZIP heteromers can stimulate transcription. A p65/CEBP- δ complex activated by LPS treatment of rabbit liver is a potent activator of serum amyloid A expression, promoting transcription from either NF- κ B or C/EBP elements within the promoter [Ray et al., 1995]. Complexes containing p50/CEBP- β and p50/CEBP- δ that strongly activate the human immunodeficiency virus type 1 (HIV-1) LTR promoter in human teratocarcinoma cells do so through interaction of only the rel component with the promoter's κ B motif [Ruocco et al., 1996], which appears to be different from that for classic NF- κ B, which requires both subunits [Kunsch et al., 1992]. Overlapping or adjacent NF- κ B/CEBP binding sites are located within the promoter regions of IL-6, IL-8,

IL-12, angiotensinogen, and serum amyloid A genes [Ray et al., 1995; Ruocco et al., 1996; Yoshimoto et al., 1996], but it is unknown whether rel/bZIP heteromers directly interact with such combined sites. C/EBP and rel proteins interact at an NF- κ B half-site in the IL-12 p40 subunit promoter to activate transcription [Plevy et al., 1997], but it remains unknown whether these proteins form heteromers in this event.

Immunoprecipitation experiments (Fig. 3) demonstrate that the nuclear concentration of heteromer increases after treatment of BMC2.3 cells with PGG-Glucan. This increase could result from translocation of preformed heteromer from the cytoplasm to the nucleus or from assembly of the heteromer after activation. Our identification of pre-formed heteromer in the cytoplasm of untreated cells (Fig. 6, lane Ctrl) favors the former hypothesis. A similar increase in the nuclear titers of p65 and p48 was also seen in the nonimmunoprecipitated nuclear extracts stimulated with PGG-Glucan (Fig. 5); however, in this case, we cannot tell whether we are monitoring heteromer-associated antigens. In these nonimmunoprecipitation immunoblots, LPS treatment probably translocates the classic NF- κ B (p65/p50), widely known to be activated in macrophage cells by this polysaccharide [for reviews see Baldwin, 1996; Sweet and Hume, 1996; Barnes and Karin, 1997], and detected by the rel-A antibody. LPS also appears to induce translocation of the p48 C/EBP- β -related protein in nonimmunoprecipitated extracts, so perhaps this antigen is a component of NF-IL-6 in these cells. For PGG-Glucan-treated extracts, the nonimmunoprecipitation Western blots are most likely, but not conclusively, responsible for monitoring heteromer translocation. Although cyclic adenosine monophosphate (cAMP) has been shown to stimulate the translocation of C/EBP- β homodimers in rat PC12 cells [Metz and Ziff, 1991], translocation of this factor after LPS treatment remains controversial [for review, see Wedel and Ziegler-Heitbrock, 1995; Akira and Kishimoto, 1997]; our data support a translocation hypothesis.

Regarding the kinases apparently used by PGG-Glucan, our data implicate PKC and PTK, but not p38 MAPK (Figs. 3, 5, 6, 7). Our current data do not allow us to determine which specific kinase isoform(s) are involved in the activation events. LPS is known to activate isoform PKC- ζ (PKC- ζ) in murine macrophage cells [Fujihara et

al., 1994], which in turn indirectly activates NF- κ B [Lozano et al., 1994], so this ζ isoform may be the PKC affected in our LPS controls. Regarding the implicated PTKs, although serine kinases have been shown to phosphorylate I κ B directly [DiDonato et al., 1997; Mercurio et al., 1997], tyrosine kinases appear to be important for NF- κ B activation in upstream events: Genistein inhibits NF- κ B activation in lymphoid cell lines [Iwasaki et al., 1992; Uckun et al., 1993] and blocks the LPS-induced activation of NF- κ B and increase in cytokine production (IL-1 β , IL-6, TNF- α) in human monocytes [Geng et al., 1993]. Some rel family members are phosphorylated on tyrosine residues [for review, see Sweet and Hume, 1996]; thus, tyrosine kinases may play a role in phosphorylating the p65 component of our heteromer complex. PKC and PTKs are part of the signaling mechanism for particulate β -glucans, which, like LPS, induce an inflammatory cytokine response, so it is not surprising that soluble β -glucans might share some signaling components with particulate β -glucans, given their chemical similarities. However, particulate zymosan (which contains both glucan and mannan components) was previously found not to upregulate NF- κ B [Shattock et al., 1994; Tran-Thi et al., 1995; Pierce et al., 1996], so zymosan may use receptors and pathways different from those used by PGG-Glucan. Zymosan has been shown to activate a unique activator protein-1 (AP-1) complex in rat Kupffer macrophage cells [Tran-Thi et al., 1995] and in human (U937) and mouse (J774) monocytic cells [Pierce et al., 1996]. Our preliminary data (not shown) indicate that PGG-Glucan may also stimulate this factor. The apparent lack of activation of p38 by PGG-Glucan is highly interesting, since this kinase is generally believed to be an important inducer of inflammatory cytokines [Lee et al., 1994], so this finding may help explain the lack of cytokine activation by PGG-Glucan.

Our immunoprecipitation experiments with anti-C/EBP- β which show that I κ B- α is associated with C/EBP- β are interesting, however we can not tell at this time whether I κ B was precipitated solely on the basis of its interaction with C/EBP- β as a dimer, or because it was bound to rel-A and p48 as a trimer. Because the dissociation could be blocked by CAL, our data expand on those of other laboratories showing that PKC activation results in removal of I κ B from classic NF- κ B [Ghosh and Baltimore, 1990; Diaz-Meco et al., 1994], even though PKC does not directly

phosphorylate I κ B- α [DiDonato et al., 1997; Mercurio et al., 1997]. Our preliminary data indicate that both LPS and PGG-Glucan activate IKK α and IKK β (E. Wakshull and D.S. Adams, unpublished observations). Because LPS signal transduction eventually results in cytokine production, while PGG-Glucan does not, activation of these kinases does not necessarily lead to cytokine production. Fine-tuned regulatory mechanisms likely exist that distinguish different I κ B-containing complexes (including the trimer described here) activated by LPS and PGG-Glucan. Participation of scaffolding proteins unique to one activator may help explain the observed differences.

In conclusion, our data are consistent with a model for PGG-Glucan activation of BMC2.3 cells in which activation of PKC and/or PTK kinases lead(s) to dissociation of I κ B- α from a rel-A/p48 heteromer, translocation of the heteromer to the nucleus, and an increase in the DNA-binding affinity of the heteromer. Because LPS treatment of BMC2.3 cells activates classic NF- κ B, while PGG-Glucan activates heteromer, the signaling pathways in these cells are different for these two polysaccharide activators.

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