Differential Expression of Peptidoglycan Recognition Protein 2 in the Skin and Liver Requires Different Transcription Factors*

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Human peptidoglycan recognition protein 2 (PGLYRP2) is an N-acetylmuramoyl-L-alanine amidase that hydrolyzes bacterial peptidoglycan and is differentially expressed in the two major organs in the human body, liver and skin. PGLYRP2 has a high constitutive expression in the liver but is not expressed in healthy human skin. PGLYRP2 mRNA is also not expressed in cultured human keratinocytes but is highly induced upon exposure to bacteria. In this study we identified the transcription start site for pglyrp2 and demonstrated that the differential expression of PGLYRP2 in hepatocytes and keratinocytes is regulated by different transcription factors whose binding sequences are located in different regions of the pglyrp2 promoter. Induction of pglyrp2 in keratinocytes is regulated by sequences in the distal region of the promoter and requires transcription factors NF-κB and Sp1, whereas constitutive expression of pglyrp2 in a hepatocyte cell line is regulated by sequences in the proximal region of the promoter and requires transcription factors c-Jun and ATF2. Regulation of constitutive and inducible expression of pglyrp2 is important for systemic and local innate immune responses to bacterial infections.

Innate immunity is an early defense mechanism against pathogens in both vertebrates and invertebrates (1, 2). The innate immune system includes pattern recognition molecules that recognize pathogens through a series of motifs called pathogen-associated molecular patterns (1, 2). Peptidoglycan recognition proteins (PGRPs)4 belong to a novel family of innate immune molecules that are highly conserved between invertebrates and vertebrates and recognize bacteria through cell wall component, peptidoglycan.

PGRPs were first identified in insects (3, 4) and then in mammals (4–7). Mammals have a family of 4 PGRPs, which were initially named PGRP-S, PGRP-L, PGRP-1α, and PGRP-1β (for “short,” “long,” or “intermediate” transcripts, respectively), by analogy to insect PGRPs (7). Recently, the Human Genome Organization Gene Nomenclature Committee changed their names to peptidoglycan recognition protein 1, 2, 3, and 4 (PGLYRP1, PGLYRP2, PGLYRP3, and PGLYRP4), and this new nomenclature is used in this article.

Mammalian PGLYRPs were originally thought to be pattern recognition receptors similar to insect PGRPs, however, recent data have demonstrated an effector role for these molecules. PGLYRP1 is produced in granulocytes and is antibacterial (7–10). PGLYRP1-deficient mice show increased sensitivity to bacterial infection and deficiency in killing of phagocytosed bacteria (8). PGLYRP2 has an N-acetylmuramoyl-L-alanine amidase activity (11, 12), which hydrolyzes the amide bond between MurNAc and l-Ala and thus removes stem peptides from the peptidoglycan molecule (13–15). PGLYRP2 produced in the liver is identical to the serum N-acetylmuramyl amidase (16). Digestion of peptidoglycan with amidase reduces or eliminates biologic activities of polymeric peptidoglycan (17, 18). PGLYRP3 and PGLYRP4 are bactericidal against several pathogenic and nonpathogenic Gram-positive bacteria, bacteriostatic against several Gram-negative bacteria, and protect mice against experimental lung infection (19).

We initially demonstrated differential and selective expression of the four PGLYRPs in various human tissues (7). Recently, we have analyzed the expression and induction of PGLYRP2 mRNA in various human tissues and cell types (20). PGLYRP2 has a high constitutive expression in the liver, low expression in some parts of the intestinal tract and cornea, and is not expressed in healthy human skin. PGLYRP2 mRNA is also not expressed in cultured human keratinocytes but is highly induced upon exposure to Gram-positive and Gram-negative bacteria. PGLYRP2 mRNA is also induced by bacteria in cultured human corneal epithelial cells, but there is no expression or induction in human primary endothelial cells and other non-epithelial cells, such as the U373 astrocytoma cell line or peripheral blood monocytes (20). The induction of PGLYRP2 correlates with expression of differentiation markers and stress response and requires activation of p38 mitogen-activated protein kinase (20).

The purpose of this study was to analyze the basis for this differential regulation of PGLYRP2 expression in hepatocytes and keratinocytes and to identify transcription factors that are involved in the constitutive expression of pglyrp2 in hepatocytes.
cytes and in the induction of pglyrp2 transcription in keratino-
cytes stimulated by bacteria.

**EXPERIMENTAL PROCEDURES**

.Cells and Cell Stimulation—Human epidermal keratinocytes, 
isolated from neonatal foreskin, were obtained from Cascade Bio-
logics (Portland, OR) and grown in EpiLife medium with Human 
Keratinocyte Growth Supplement. These cells were used between 
the 3rd and 5th passage, and before stimulation the cells were main-
tained in medium without supplement for 18–24 h as described 
previously (20). The hepatocyte cell line C3A (ATCC, Rockville, 
MD) and the human embryonic kidney cell line HEK293 stably 
expressing Toll-like receptor 2 (TLR2) (21) were cultured in 
Dulbecco’s modified Eagle’s medium with 10% defined fetal calf serum.

Keratinocytes and HEK293 cells were stimulated with 2 × 
10⁶ cells/ml of heat-killed (70 °C, 30 min) Bacillus subtilis 
(ATCC 6633) or Enterobacter cloacae (ATCC 13047). C3A cells 
were not stimulated for most experiments because our initial 
data showed that exposure of C3A cells to bacteria (as above) 
did not increase the already high constitutive expression of 
PGLYRP2 in these cells.

**RNA and Real Time Reverse Transcriptase-PCR**—RNA from 
normal human liver and skin were obtained from Clontech 
(Palo Alto, CA) and Stratagene (La Jolla, CA), respectively. RNA 
further from cultured keratinocytes and C3A cells was isolated with the 
TRIzol reagent (Invitrogen). In experiments with the calpain 
inhibitor ALLN (Calbiochem, La Jolla, CA), keratinocytes were 
cultured with the inhibitor (100 μM) for 1 h before stimulation.

Quantitative real time reverse transcriptase-PCR was done 
using TaqMan reagents and the ABI Prism 7000 Sequence 
Detection System as described previously (20). For quantitative 
real time PCR, the comparative cycle threshold method was 
used with 18 S RNA as an endogenous control.

**Mapping the Transcription Start Site for pglyrp2**—The 
transcription start site for pglyrp2 was mapped by two different 
methods, primer extension and 5’ RACE cloning. Primer extension 
was done using the Primer Extension System from Promega. Total 
RNA was isolated from 3 × 10⁶ stimulated and unstimulated kera-
tinocytes and from unstimulated C3A cells using the RNeasy Purifi-
cation Kit (Qiagen, Valencia, CA). The primer used for extension 
(Table 1) was labeled with [γ-³²P]ATP. 100 fmol of labeled primer 
was annealed to 20 μg of RNA and used for the synthesis of cDNA 
as per the manufacturer’s instructions. A sequencing reaction 
was run in parallel using the same primer and clone 
P2(−2005)Luc for a template using Sequenase version 2.0 
(United States Biochemicals) and labeled with [α-³²P]dATP. 
The products were analyzed on an 8% denaturing polyacrylam-
ide–sequencing gel and then visualized by autoradiography.

The 5’ end of PGLYRP2 mRNA was also mapped by 5’ RACE 
using liver Marathon-Ready cDNA from Clontech. The 5’ 
RACE reactions were done as per the manufacturer’s instruc-
tions. Briefly, the 5’ RACE PCR was done using a gene-specific 
primer (Table 1) and an adapter-specific primer (Clontech). 
The amplified fragments were cloned into a T.A vector (Invitro-
gen), analyzed by restriction digestion, and sequenced. More 
than 30 clones were analyzed for each primer.

**Differential Regulation of Peptidoglycan Recognition Protein 2**

**Table 1**

| Name | Sequence 5’ → 3’ |
|------|-----------------|
| P2(−16)R | TTCCAGAGGGATATTTCTGTTTG |
| P2(+514)R | CTGGAGAGGGATCTTTCCTAC |

**pglyrp2 Promoter Constructs**—The pglyrp2 promoter, −2005 to −15 bp upstream of the start codon, was amplified by PCR 
from genomic DNA and cloned into the KpnI and Nhel sites of 
the luciferase reporter vector pGL3 basic (Promega). This clone 
was referred to as P2(−2005)Luc. The promoter region was 
sequenced and aligned with the human genomic sequence in 
GenBank™ (AC011492). All deletion constructs were made 
using TaqMan reagents and the ABI Prism 7000 Sequence 
Detection System as described previously (20). For quantitative 
real time PCR, the comparative cycle threshold method was 
used with 18 S RNA as an endogenous control.
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Activation of pglyrp2 Promoter—Keratinocytes, C3A, and HEK293 cells were cultured at 0.2 × 10^6 cells/ml in 24-well plates (0.5 ml/well) in complete medium. Subconfluent cells were transfected in duplicate with the different pglyrp2 promoter-luciferase reporter plasmids or empty vector (pLuc) at 0.8 μg/ml for keratinocytes and 0.2 μg/ml for C3A and HEK293 cells. In co-transfection experiments keratinocytes were transfected with P2(-2005)Luc and 0.03 μg/ml of a control vector or the constitutive repressor of IκB, IκBΔN (21), or with NFκB-Luc (21) and the dominant negative forms of p38 or ERK1 (20). C3A cells were transfected with P2(-607)Luc and 0.03 μg/ml of a control vector or the dominant negative forms of ATF2 (23) or c-Jun (24). All transfections were done using Lipofectamine 2000 (Invitrogen). Following transfection keratinocytes were maintained in medium without supplement for 18–24 h and then stimulated with bacteria (as above) for 12 h and cell lysates were prepared (20). HEK293 cells were stimulated with bacteria (as above) for 12 h immediately following transfection and cell lysates were prepared (21). C3A cells were not stimulated and cells were harvested 18–24 h after transfection. Expression of the pglyrp2 gene (activation of the pglyrp2 promoter) and NF-κB-regulated transcription was measured as luciferase activity and determined in the cell lysates as described previously (22). The results are expressed as a direct count of luciferase activity, or as percent of the P2(-2005)Luc, P2(-281)Luc, P2(-607)Luc, or NFκB-Luc constructs.

Electrophoretic Mobility Shift Assay—Keratinocytes and C3A cells were cultured at 0.35–0.4 × 10^6 cells/ml in 24-well plates (1.0 ml/well) for 16–20 h. Keratinocytes were stimulated as described above and in the figure legends. Cells were harvested and nuclear extracts were prepared as described previously (25). Oligonucleotides homologous to specific regions in the pglyrp2 promoter (shown in Table 1) were labeled with [α-32P]dATP and incubated with nuclear proteins for 30 min at 22 °C (25). To identify the protein(s) that binds an oligonucleotide, nuclear extracts were preincubated with specific antibodies for 30 min at 22 °C followed by incubation with the labeled oligonucleotide. Antibodies to p50, p65, and c-Rel were from Upstate Biotechnology (Lake Placid, NY) and antibodies to Sp1, c-Jun, and ATF2 were from Santa Cruz Biotechnology. To determine specificity of binding, samples were preincubated with an excess of unlabeled specific or nonspecific oligonucleotide for 20 min at 22 °C. All samples were then separated on 5% nondenaturing polyacrylamide gels and the DNA-protein complexes were visualized by autoradiography (25).

Chromatin Immunoprecipitation (ChIP)—The ChIP assay was performed using a kit from Upstate Biotechnology as per the manufacturer’s instructions. Briefly, keratinocytes and C3A cells were cultured at 1 × 10^6 cells/ml in 10-cm tissue culture plates (12 ml/plate). One group of keratinocytes was stimulated for 25 min with Enterobacter and another group was not stimulated. The DNA was fixed with 1% formaldehyde in Dulbecco’s phosphate-buffered saline for 15 min at room temperature and then treated with 1 M glycine for 10 min at room tempera-

ture. The cells were harvested and lysed in SDS lysis buffer and sonicated 4 × 20 s with 10 min on ice between each sonication. The DNA fragments were analyzed on a gel and were 200 to 1000 bp in size. Cell lysates were pre-cleared with salmon sperm DNA/protein A-agarose for 30 min at 4 °C and stored in aliquots at 80 °C. Aliquots were used for immunoprecipitation with antibodies against p65, Sp1, c-Jun, ATF2, or mock immunoprecipitated. Input DNA was not immunoprecipitated. The protein-DNA complexes were heated at 65 °C for 4 h to reverse the cross-linking and DNA was purified using the Qiagen PCR purification kit (Qiagen). Regions of the pglyrp2 promoter that contained the NF-κB, Sp1, or AP-1 sites were amplified using the primers listed in Table 1. Primers that amplified a different region of the promoter were used as control (Table 1).

Degradation of IκB and Western Blots—Keratinocytes were cultured and stimulated as described above for electrophoretic mobility shift assay. Whole cell lysates were prepared, separated on 12% SDS-PAGE, and transferred to Immobilon P as described previously (25). Degradation of IκB was determined by Western blotting with an antibody specific to IκB (Santa Cruz Biotechnology).

RESULTS

Mapping of the Transcription Start Site of PGLYRP2 mRNA and Analysis of the pglyrp2 Promoter—We have previously shown that PGLYRP2 mRNA is differentially expressed in various human tissues (20). To identify the regulatory elements and transcription factors that are involved in this complex expression of PGLYRP2 we first mapped the 5’ end of the PGLYRP2 mRNA by two different methods: primer extension and 5’ RACE reaction. Primer extension with RNA from keratinocytes and C3A cells gave two products that mapped the 5’ end to G at 107 bp or A at 102 bp upstream of the start codon (Fig. 1A). Primer extension products with keratinocytes stimulated with bacteria were more prominent than the products obtained with unstimulated keratinocytes (Fig. 1A). This increase indicates the induction of PGLYRP2 mRNA in keratinocytes exposed to bacteria. RNA from unstimulated C3A cells also had strong primer extension products, which indicates the high constitutive expression of PGLYRP2 in liver. The 5’ RACE reactions using liver Marathon Ready cDNA resulted in clones with 5 different 5’ ends (Fig. 1B). One of these 5’ ends was identical to the G that was mapped as a 5’ end in the primer extension experiment and this G at the −107 bp position is considered the transcription start site of PGLYRP2 mRNA and the region upstream as the promoter.

We analyzed about 2000 bases upstream of the translation start site using both Match and Patch programs in the Transfac 7.0 data base (Biobase). The pglyrp2 promoter does not have a TATA or a CAAT box, however, numerous potential transcription factor-binding sites were identified. Putative sites on the distal promoter included 2 NF-κB, Sp1, C/EBP, AP-2, and NF-1, whereas the proximal promoter had several AP-1 binding elements (Fig. 1B).

PGLYRP2 mRNA and pglyrp2 Promoter-Luciferase Plasmids Show Differential Expression in the Liver and Skin, and This Requires Different Regions of the pglyrp2 Promoter—We have previously shown and confirmed in this study that PGLYRP2 mRNA was differentially expressed in various organs (20). To identify the regulatory elements and transcription factors that are involved in this complex expression of PGLYRP2 we first mapped the 5’ end of the PGLYRP2 mRNA by two different methods: primer extension and 5’ RACE reaction. Primer extension with RNA from keratinocytes and C3A cells gave two products that mapped the 5’ end to G at 107 bp or A at 102 bp upstream of the start codon (Fig. 1A). Primer extension products with keratinocytes stimulated with bacteria were more prominent than the products obtained with unstimulated keratinocytes (Fig. 1A). This increase indicates the induction of PGLYRP2 mRNA in keratinocytes exposed to bacteria. RNA from unstimulated C3A cells also had strong primer extension products, which indicates the high constitutive expression of PGLYRP2 in liver. The 5’ RACE reactions using liver Marathon Ready cDNA resulted in clones with 5 different 5’ ends (Fig. 1B). One of these 5’ ends was identical to the G that was mapped as a 5’ end in the primer extension experiment and this G at the −107 bp position is considered the transcription start site of PGLYRP2 mRNA and the region upstream as the promoter.

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mRNA is differentially expressed in the liver and skin (Fig. 2A). To determine the mechanisms that regulate this differential expression between the liver and skin we analyzed the expression of PGLYRP2 mRNA in the C3A human liver cell line and in primary human keratinocytes. C3A cells have high constitutive expression of PGLYRP2 mRNA, which did not change in the presence of bacteria (Fig. 2B). In contrast, keratinocytes do not express PGLYRP2 mRNA, however, stimulation of cells by Bacillus or Enterobacter induced high expression (Fig. 2B) as shown previously (20).

To identify regions of the promoter that regulate the expression of PGLYRP2 in keratinocytes and C3A cells we constructed pglyrp2 promoter-luciferase plasmids. We first made plasmids with about 2 kbp (P2(H110022005)Luc) or 1 kbp (P2(H110021010)Luc) upstream regions of the start codon and these plasmids were transfected into keratinocytes, C3A cells, and HEK293 cells and pglyrp2 transcription was determined by measuring luciferase activity. Both P2(H110022005)Luc and P2(H110021010)Luc plasmids were highly expressed in C3A cells (Fig. 2C) and the expression did not change in the presence of bacteria (data not shown). These results indicate that the regulatory elements required for high constitutive expression of PGLYRP2 in the liver lie within the 1-kbp region of the promoter and we have termed this the proximal promoter. Neither construct is expressed or induced in HEK293 embryonic kid-

![Diagram of the transcription start site of human PGLYRP2 mRNA and sequence and analysis of the pglyrp2 promoter. A, the 5' end of PGLYRP2 mRNA was determined by primer extension analysis of total RNA from C3A hepatocytes and keratinocytes. Keratinocytes were not stimulated (Ent) or stimulated (Ent) for 4 h with Enterobacter. The adjacent lanes G, A, T, and C represent DNA sequencing reactions with the P2(H11002) plasmid and the same primer as used for primer extension. The sequence of the complementary strand is shown. B, the nucleotide sequence of the upstream region of the pglyrp2 gene is shown. Nucleotides are numbered relative to the start codon with A of ATG (underlined bold) being +1. The 5' ends of PGLYRP2 mRNA identified by primer extension are represented by an asterisk and bold letters. The 3' ends and the 3' end of the various fragments of the pglyrp2 promoter that were cloned are indicated by closed triangles pointing up or down, respectively. Potential transcription factor binding sites are shown by a line either above the sequence or below the sequence and by the name of the transcription factor.
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Because constitutive expression of PGLYRP2 in C3A cells requires the 1-kb region of the promoter we tested the expression of pglyrp2 promoter luciferase plasmids with serial deletions within this region of the promoter. The P2(−281)Luc construct is the highest expressed in C3A cells (Fig. 3C). The sequence upstream of −281 bp may contain repressor elements indicated by lower expression of the P2(−1010)Luc construct as compared with the P2(−281)Luc construct. The region of the promoter between −281 and −157 bp is required for the maximal expression of pglyrp2 in C3A cells, however, sequences in the 5′-untranslated region of PGLYR2 mRNA control the low expression of PGLYRP2 mRNA (see P2(−100)Luc construct). Expression of all plasmids in C3A cells was constitutive and did not change in the presence of bacteria (data not shown). These results indicate that different regions of the pglyrp2 promoter are involved in up- and down-regulation of pglyrp2 transcription in C3A cells. In this study we focused on the region between −281 and −157 bp of the pglyrp2 promoter, because this region is required for the maximal expression of PGLYRP2 mRNA in C3A cells.

**Nuclear Proteins from Keratinocytes Stimulated with Bacteria Bind NF-κB Consensus Sequences in the pglyrp2 Promoter**—The two regions of pglyrp2 promoter required for maximal induction of PGLYRP2 mRNA contain binding sites for several different transcription factors, including NF-κB and Sp1. NF-κB is a key transcription factor involved in the regulation of immune responses and we next determined whether NF-κB is involved in the regulation of expression of pglyrp2. The pglyrp2 promoter has 2 NF-κB sites, κB1 at −1082 bp and κB2 at −1057 bp upstream of the start codon (Fig. 1B). Oligonucleotides spanning these two binding sites were used for determining the binding of proteins by the electrophoretic mobility shift assay. Nuclear extracts from unstimulated keratinocytes do not contain proteins that bind κB1 or κB2 sites (Fig. 4A). However, nuclear extracts from keratinocytes stimulated with Enterobacter or Bacillus contained proteins that bind both κB1 and κB2 sites (Fig. 4A). Activation of proteins that bind these sites is rapid and within 10 min strong binding is seen in both Enterobacter- and Bacillus-stimulated cells.

The specificity of binding of nuclear proteins from stimulated keratinocytes to the κB1 and κB2 sequences was con-

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**FIGURE 2.** PGLYRP2 is differentially expressed in the liver and skin, and this requires different regions of the pglyrp2 promoter. A and B, PGLYRP2 mRNA expression was analyzed by real time reverse transcriptase-PCR in liver and skin (A), and in C3A hepatocytes and keratinocytes stimulated with Bacillus or Enterobacter or medium (Nil) (B). C and D, C3A, keratinocytes, and HEK293 cells expressing TLR2 were transfected with the pglyrp2 promoter luciferase plasmids, P2(−2005)Luc and P2(−1010)Luc or with the empty vector, pLuc, and not stimulated (C), or stimulated with Bacillus, Enterobacter, or medium (Nil) (D), and pglyrp2 transcription was measured as luciferase activity. The results are means of four experiments (A and B) or a representative experiment of four to five similar experiments (C and D).

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ney cells expressing TLR2 (Fig. 2, C and D), which respond to stimulation with bacteria by activating NF-κB and inducing interleukin-8 mRNA expression (21).

To narrow down the region of the distal promoter that is required for the induction of PGLYRP2 expression in keratinocytes stimulated with bacteria, we next tested the expression of pglyrp2 promoter luciferase plasmids with serial deletions within the 1−2-kbp upstream region (Fig. 3). These plasmids were not expressed in unstimulated keratinocytes but were induced upon exposure to bacteria, however, the induction is reduced and eventually lost as the promoter is deleted to 1 kbp (see P2(−1010)Luc construct). Two regions within the distal promoter were necessary for the induction of pglyrp2 transcription in keratinocytes stimulated with bacteria (Fig. 3B). The first region is between −1198 and −1010 bp and the second is between −1809 and −1604 bp on the promoter (Fig. 3B). Numerous putative transcription factor binding sites are located within these two regions of the pglyrp2 promoter, including, NF-κB, Sp1, AP1, and AP2 (Figs. 1 and 3).
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A

\[
\begin{align*}
\text{Sp-1} & \quad \text{C/EBP} \\
-2005 & \quad -1809 & \quad -1604 & \quad -1405 \\
\text{Sp-1} & \quad \text{GATA} & \quad \text{AP1} & \quad \text{Sp-1} \\
-1198 & \quad -1010 & \quad -100 & \quad +1 \\
\text{CREB} & \quad \text{CETS} & \quad \text{AP2} & \quad \text{NF-kB} \\
-400 & \quad -281 & \quad -260 & \quad -249 \\
\end{align*}
\]

B

**Keratinocytes**

**C3A cells**

![Graphs showing PGLYRP2 transcription induction in keratinocytes and C3A cells](image)

**FIGURE 3.** *pglyrp2* transcription induction by bacteria in keratinocytes requires distal regions, whereas constitutive expression in C3A hepatocytes requires proximal regions of the *pglyrp2* promoter. A, summary of the potential binding sites for different transcription factors in the *pglyrp2* promoter. The closed triangle and the numbers below indicate the 5' ends of fragments of the *pglyrp2* promoter that were cloned upstream of the luciferase gene. The arrow indicates the transcription start site. B and C, keratinocytes and C3A cells were transfected in duplicate samples with the indicated *pglyrp2* promoter luciferase plasmids, and keratinocytes were stimulated with *Enterobacter*. Transcription of *pglyrp2* was measured as luciferase activity. The results are means of four experiments (B and C).

Confirmed using excess unlabeled specific and nonspecific oligonucleotides (Fig. 4B). The binding of nuclear proteins to both the κB1 and κB2 sequences was inhibited by an excess of unlabeled specific oligonucleotide, with a κB1 or a κB2 site, respectively, but not by nonspecific oligonucleotide with no NF-κB binding element (Fig. 4B). These results indicate that the binding of nuclear proteins from keratinocytes stimulated with bacteria to the κB1 and κB2 sites on the *pglyrp2* promoter is specific.

To identify the specific proteins that bind the κB1 and κB2 sequences on the PGLYRP2 promoter, we performed the binding assay in the presence of Abs against different members of the NF-κB family, p50, p65, and c-Rel, and nonspecific IgG. Two different Abs against p65 were used. Abs against p50 and p65 but not c-Rel or nonspecific IgG abolished the binding of nuclear proteins to both the κB1 and κB2 sequences on the *pglyrp2* promoter (Fig. 4C). These results indicate that *Enterobacter* stimulates keratinocytes and induces the activation of p50- and p65-containing NF-κB dimers that can bind both κB1 and κB2 sites on the *pglyrp2* promoter.

We next determined whether p65 is recruited to the endogenous *pglyrp2* promoter in vivo in stimulated keratinocytes using the chromatin immunoprecipitation assay. Stimulation of keratinocytes with *Enterobacter* induced strong recruitment of p65 to the *pglyrp2* promoter that was not observed in unstimulated keratinocytes, demonstrated by the 159-bp amplified fragment in the stimulated sample (Fig. 4D, +Ent, +Ab, NF-κB ChIP primers). Immunoprecipitation by anti-p65 Ab was specific because there was no amplification in the mock immunoprecipitated sample (−Ab lane). Amplification of the p65 binding region is also specific because there was no amplification of an unrelated region of the promoter (Fig. 4D, Control ChIP primers). Input DNA was used as a control to demonstrate amplification of the *pglyrp2* promoter with both sets of primers before immunoprecipitation. The amplified product with the control ChIP primers is 146 bp.

The inactive form of p65 is sequestered in the cytoplasm bound to its inhibitory protein, IkB and activation of p65 requires degradation of IkB. Activation of p65 in keratinocytes stimulated with *Enterobacter* was accompanied by transient degradation of IkB in the cytoplasm (Fig. 4E). In unstimulated cells there was no degradation of IkB. These results confirm the binding of NF-κB to the *pglyrp2* promoter in vivo and in vitro and demonstrate that the NF-κB dimer is composed of p50 and p65 proteins.

**Bacteria-induced Transcription of pglyrp2 in Keratinocytes Requires NF-κB**—We next determined whether active NF-κB is required for the induced transcription of *pglyrp2* in keratinocytes stimulated with bacteria. We first tested for the effect of the calpain inhibitor I, ALLN, on induction of *pglyrp2* transcription. ALLN inhibits the calpain family of proteases, which results in the inhibition of degradation of IkB and consequently the activation of NF-κB. ALLN almost completely abolished the induction of PGLYRP2 mRNA in keratinocytes stimulated with both *Enterobacter* and *Bacillus* (Fig. 5A).

We then confirmed the requirement for the degradation of IkB in the activation of p65 and the expression of *pglyrp2*. A constitutive repressor of IkB, IkBΔN, was used in cotransfection experiments with the P2(−2005)Luc plasmid. IkBΔN, but not the empty vector, almost completely eliminated the induced expression of *pglyrp2* (Fig. 5B).

We next determined whether binding of NF-κB to the κB1 and/or κB2 sites on the *pglyrp2* promoter is required for the induced transcription of *pglyrp2* in keratinocytes stimulated with bacteria. P2(−2005)Luc plasmids with non-functional κB1

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**FIGURE 3.** *pglyrp2* transcription induction by bacteria in keratinocytes requires distal regions, whereas constitutive expression in C3A hepatocytes requires proximal regions of the *pglyrp2* promoter. A, summary of the potential binding sites for different transcription factors in the *pglyrp2* promoter. The closed triangle and the numbers below indicate the 5' ends of fragments of the *pglyrp2* promoter that were cloned upstream of the luciferase gene. The arrow indicates the transcription start site. B and C, keratinocytes and C3A cells were transfected in duplicate samples with the indicated *pglyrp2* promoter luciferase plasmids, and keratinocytes were stimulated with *Enterobacter*. Transcription of *pglyrp2* was measured as luciferase activity. The results are means of four experiments (B and C).
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A—C, nuclear extracts from stimulated keratinocytes were incubated with radioactively labeled oligonucleotides homologous to the pglyrp2 promoter with binding sites for NF-κB1 or NF-κB2. A, keratinocytes were stimulated with Bacillus (Bac) or Enterobacter (Ent) for the indicated times (min) or not stimulated (N). B and C, keratinocytes were stimulated with Enterobacter for 30 min. Nuclear extracts were preincubated with increasing amounts of unlabeled specific (κB1 or κB2) or nonspecific (NS) oligonucleotides (B) or with the indicated Abs (C) before the addition of the radioactively labeled oligonucleotide. D, keratinocytes were stimulated with Enterobacter (+Ent) for 25 min or not stimulated (−Ent), and chromatin immunoprecipitation was performed. Samples immunoprecipitated with anti-p65 Ab or mock immunoprecipitated and Input DNA were analyzed with two sets of primers, NF-κB ChIP and Control ChIP primers. The arrows indicate the specifically amplified products. E, keratinocytes were stimulated with Bacillus (Bac) or Enterobacter (Ent) for the indicated times (min) and cell lysates were separated by SDS-PAGE and analyzed by Western blots for degradation of IκB using an anti-κB Ab. The results are from one of three similar experiments (A–E).

FIGURE 4. NF-κB binds to two sites on the pglyrp2 promoter in keratinocytes stimulated with bacteria. A–C, nuclear extracts from stimulated keratinocytes were incubated with radioactively labeled oligonucleotides homologous to the pglyrp2 promoter with binding sites for NF-κB1 or NF-κB2. A, keratinocytes were stimulated with Bacillus (Bac) or Enterobacter (Ent) for the indicated times (min) or not stimulated (N). B and C, keratinocytes were stimulated with Enterobacter for 30 min. Nuclear extracts were preincubated with increasing amounts of unlabeled specific (κB1 or κB2) or nonspecific (NS) oligonucleotides (B) or with the indicated Abs (C) before the addition of the radioactively labeled oligonucleotide. D, keratinocytes were stimulated with Enterobacter (+Ent) for 25 min or not stimulated (−Ent), and chromatin immunoprecipitation was performed. Samples immunoprecipitated with anti-p65 Ab or mock immunoprecipitated and Input DNA were analyzed with two sets of primers, NF-κB ChIP and Control ChIP primers. The arrows indicate the specifically amplified products. E, keratinocytes were stimulated with Bacillus (Bac) or Enterobacter (Ent) for the indicated times (min) and cell lysates were separated by SDS-PAGE and analyzed by Western blots for degradation of IκB using an anti-κB Ab. The results are from one of three similar experiments (A–E).

(kB1m) or κB2 sites (κB2m) or with functional κB1/2 sites (wt) were tested for expression in transfected keratinocytes stimulated with bacteria. Mutations within κB1 or κB2 sites on the pglyrp2 promoter almost completely eliminated the induced expression of pglyrp2 (Fig. 5C). These results indicate that both κB1 and κB2 are required and are equally important for the induction of pglyrp2 in keratinocytes stimulated with bacteria.

In our previous results we have shown activation of p38 mitogen-activated protein kinase and inhibition of PGLYRP2 expression by both dominant negative p38 and SB203580, a specific inhibitor of p38 activation, in keratinocytes stimulated with bacteria. Here we tested the role of p38 mitogen-activated protein kinase in the activation of NF-κB. Dominant negative p38 but not dominant negative ERK inhibited NF-κB activation in keratinocytes exposed to bacteria (Fig. 5D). These data indicate that activation of p38 results in the activation of NF-κB and transcription of pglyrp2.

Nuclear Proteins from Keratinocytes Stimulated with Bacteria Bind an Sp1 Site on the pglyrp2 Promoter and this Site Is Required for Induced Transcription of pglyrp2—The second region important in the regulation of expression of PGLYRP2 in keratinocytes exposed to bacteria is between −1809 and −1604 bp upstream of the start codon (Fig. 3B). This region has binding sites for several transcription factors, including Sp1 at −1802 bp (Fig. 1B). We next determined the role of Sp1 in transcription of pglyrp2 in keratinocytes stimulated with bacteria. An oligonucleotide spanning this Sp1 binding site was used to determine binding of nuclear proteins from keratinocytes by the gel shift assay. Nuclear extracts from keratinocytes stimulated with Enterobacter but not from unstimulated keratinocytes contained proteins that bind Sp1 (Fig. 6A). Binding to the Sp1 sites was rapid and transient, the binding was strong within 10 min and it was gone by 90 min.

The binding of nuclear proteins to the Sp1 site was inhibited by an excess of unlabeled specific oligonucleotide (Sp1), but not by nonspecific oligonucleotide with no Sp1 binding element (Fig. 6B). These results indicate that the binding of nuclear proteins, from keratinocytes stimulated with bacteria to the Sp1 site on the pglyrp2 promoter is specific.

To identify the specific proteins that bind the Sp1 sequence on the pglyrp2 promoter we performed the binding assay in the presence of anti-Sp1 Ab and nonspecific IgG. The Ab against Sp1 protein, but not IgG, abolished the binding of nuclear proteins to the Sp1 consensus sequence on the pglyrp2 promoter (Fig. 6C). These results indicate that Enterobacter stimulates keratinocytes and induces binding of the Sp1 protein to the Sp1 site on the pglyrp2 promoter.
Differential Regulation of Peptidoglycan Recognition Protein 2

We next determined using the chromatin immunoprecipitation assay whether Sp1 is recruited to the endogenous PGLYRP2 promoter in stimulated keratinocytes. Sp1 recruitment to the promoter was significantly enhanced in Enterobacter-stimulated keratinocytes compared with unstimulated keratinocytes, as demonstrated by higher levels of the 237-bp fragment amplified in the stimulated sample (Fig. 6D, +Ent, +Ab, Sp1 ChIP primers). Immunoprecipitation by the anti-Sp1 Ab was specific because there was no amplification in the mock immunoprecipitated sample (−Ab lane). Amplification of the Sp1 binding region (Fig. 4D, Sp1 ChIP primers) was also specific because there was no amplification of an unrelated region of the promoter (Fig. 4D, Control ChIP primers). Input DNA was used as a control to demonstrate amplification of the PGLYRP2 promoter with both sets of primers before immunoprecipitation. The amplified product with the control ChIP primers is 146 bp.

We next determined whether binding of Sp1 to the Sp1 site is required for the induced expression of pglyrp2 in keratinocytes stimulated with bacteria. P2(−2005)Luc plasmids with the non-functional Sp1 site or with the functional Sp1 (wt) binding element were tested for expression in transfected keratinocytes stimulated with bacteria. Mutations within the Sp1 binding site of the promoter reduced the induced expression of pglyrp2 by 40% (Fig. 6E). These results indicate that the Sp1 site is required for the full transcription of pglyrp2 in keratinocytes stimulated with bacteria.

ATF2 and c-Jun Bind to an AP-1 Site on the pglyrp2 Promoter and This Site Is Required for Maximum Constitutive Transcription of pglyrp2 in C3A Liver Cells—The region between −281 and −157 bp of the pglyrp2 promoter is required for the maximum expression of PGLYRP2 in the C3A hepatocyte cell line, and this region contains putative binding sites for several different transcription factors including two AP-1 binding motifs. We next determined whether nuclear proteins from C3A cells bind both Sp1 and/or the Sp-1 consensus sequence using double-stranded oligonucleotides spanning these regions of the promoter. Binding of nuclear proteins to the AP-1 site at the −201 position and the

FIGURE 5. NF-κB is required for the induction of pglyrp2 transcription in keratinocytes stimulated with bacteria and is activated by p38. A, keratinocytes were preincubated for 1 h in medium alone, with Me₂SO (DMSO) vehicle, or ALLN and then stimulated with Bacillus or Enterobacter for 4 h. PGLYRP2 mRNA expression was analyzed by real-time reverse transcriptase-PCR. B—D, keratinocytes were transfected with the indicated plasmids, stimulated with Enterobacter, and transcription of luciferase regulated by PGLYRP2 promoter (B and C) or with the functional Sp1 (wt) binding region (Fig. 4D, Sp1 ChIP primers, −Ent, +Ent, Input). Nuclear extracts from stimulated keratinocytes were incubated with radioactively labeled oligonucleotide homologous to the pglyrp2 promoter with a binding site for Sp1. The results are from one of three similar experiments (A–D).

FIGURE 6. Sp1 binds to the pglyrp2 promoter in keratinocytes stimulated with bacteria and this binding is required for the induction of pglyrp2 transcription. A–C, nuclear extracts from stimulated keratinocytes were incubated with radioactively labeled oligonucleotide homologous to the pglyrp2 promoter with a binding site for Sp1. A, keratinocytes were stimulated with Enterobacter (Ent) for the indicated times (min) or not stimulated (N). B and C, keratinocytes were stimulated with Enterobacter for 30 min. Nuclear extracts were preincubated with increasing amounts of unlabeled specific (Sp1) or nonspecific (NS) oligonucleotides or with the indicated Abs (C) before the addition of the radioactively labeled oligonucleotide. D, keratinocytes were stimulated as in Fig. 4D, and chromatin immunoprecipitation was performed. Samples immunoprecipitated with anti-Sp1 Ab, mock immunoprecipitated, and Input DNA were analyzed with two sets of primers, Sp1 ChIP and Control ChIP primers. The arrows indicate the amplified products. E, keratinocytes were transfected with the indicated plasmids, stimulated with Enterobacter, and transcription of pglyrp2 was measured as luciferase activity. The results are from one of three similar experiments (A–D) or the means of four experiments (E). wt, wild type.
Differential Regulation of Peptidoglycan Recognition Protein 2

FIGURE 7. ATF2 and c-Jun bind to an AP-1 site on the pglyrp2 promoter in C3A hepatocytes and this site is required for the constitutive expression of pglyrp2. A and B, C3A nuclear extracts were incubated with radioactively labeled oligonucleotide homologous to the pglyrp2 promoter with the binding site for AP-1. Nuclear extracts were preincubated with increasing amounts of unlabeled specific (AP1) or nonspecific (NS) oligonucleotides (A) or with the indicated Abs (B) before the addition of the radioactively labeled oligonucleotide. C, chromatin immunoprecipitation was performed with C3A cells. Samples immunoprecipitated with anti-ATF2 Ab, anti-c-Jun Ab, or mock immunoprecipitated and Input DNA were analyzed with two sets of primers, AP1 ChIP and Control (Ctrl) ChIP primers. The arrows indicate the amplified products. D and E, C3A cells were transfected with the indicated plasmids and pglyrp2 transcription was measured as luciferase activity. The results are one of three similar experiments (A–C), the means of four experiments (D), or two experiments (E).

Sp-1 site was nonspecific (data not shown) and no further studies were done on these putative binding sites.

Nuclear proteins from C3A cells bound to the AP-1 sequence at the −260-bp position and the binding was eliminated with an excess of unlabeled specific oligonucleotide but not by the nonspecific oligonucleotide with no AP-1 binding element (Fig. 7A). We next identified the specific proteins in C3A cells that bound to this AP-1 on the pglyrp2 promoter. Antibodies to different members of the AP-1 and CREB families and to ER, retinoid X receptor, and peroxisome proliferator-activated receptor-α proteins were tested in gel shift experiments. We included antibodies to ER, retinoid X receptor, and peroxisome proliferator-activated receptor-α, because the sequence in the AP-1 motif at the −260-bp position is also identified as the ER(retinoid X receptor binding element. Antibodies against ATF2 and c-Jun caused a shift in the binding pattern of nuclear proteins to the AP-1 motif (Fig. 7B), but antibodies to CREB, ATF-1, c-Fos, JunB, ER, retinoid X receptor, and peroxisome proliferator-activated receptor-α did not change the binding pattern (data not shown). These data indicate that transcription factors ATF2 and c-Jun in nuclear extracts from C3A cells bind specifically to the AP-1 consensus sequence at the −260-bp position on the pglyrp2 promoter.

We next determined using the chromatin immunoprecipitation assay whether c-Jun and ATF2 are recruited to the endogenous pglyrp2 promoter in vivo in C3A cells. Both c-Jun and ATF2 were constitutively bound to the promoter in C3A cells demonstrated by the 316-bp fragment in the sample amplified with the AP1 primers (Fig. 7C, +Ab, AP1 primers). Immunoprecipitation with the anti-c-Jun Ab and the anti-ATF2 Ab was specific because there was no amplification in the mock immunoprecipitated sample (−Ab lane). Amplification of the AP1 binding region (Fig. 7C, AP1 ChIP primers) was also specific because there was no amplification of an unrelated region of the promoter (Fig. 7C, Control ChIP primers). Input DNA was used as a control to demonstrate amplification of the pglyrp2 promoter with both sets of primers before immunoprecipitation. The amplified product with the control ChIP primers was 146 bp.

We next determined whether this AP-1 site is required for maximum constitutive expression of PGLYRP2 in C3A cells. To test for AP-1 requirement we compared the levels of expression of the pglyrp2 promoter luciferase plasmids, P2(−260)Luc and P2(−249)Luc to P2(−281)Luc, the highest expressing plasmid in C3A cells (Fig. 3D). The P2(−260)Luc plasmid contains the AP-1 consensus sequence, whereas the P2(−249)Luc is 12 bp smaller and lacks the AP-1 binding site. A deletion of just the AP-1 binding site reduced the constitutive expression of PGLYRP2 mRNA by 30% (P2(−249)Luc compared with P2(−260)Luc) (Fig. 7D). The P2(−260)Luc plasmid also had lower expression than the P2(−281)Luc plasmid (Fig. 7D, 34% lower), however, we were unable to identify putative binding sites within this region of the promoter either by TRANSFAC analysis or by gel shift assays. These results indicate that the AP-1 binding site and binding of c-Jun and ATF2 to the pglyrp2 promoter are required for the maximum constitutive transcription of pglyrp2 in C3A cells.

To confirm the role of ATF2 and c-Jun in the constitutive expression of pglyrp2 in C3A cells, we determined the effect of dominant negative forms of these proteins. Both, dominant negative ATF2 and c-Jun inhibited pglyrp2 transcription in
C3A cells (Fig. 7E), thus, confirming that ATF2 and c-Jun are two transcription factors required for maximum constitutive expression of *pglyrp2* in C3A cells.

**DISCUSSION**

In this study we have identified the transcription start site for human PGLYRP2 mRNA, characterized the *pglyrp2* promoter, and identified the regulatory elements that are involved in the differential expression of the *pglyrp2* gene. PGLYRP2 is not expressed in normal human skin or in uninduced cultured keratinocytes, however, it is strongly induced in keratinocytes upon exposure to bacteria. In contrast, PGLYRP2 is constitutively expressed in the liver and the C3A hepatocyte cell line, and the level of expression does not change upon exposure to bacteria. Our results demonstrate that induction in keratinocytes is completely dependent on sequences within the distal part of the promoter, whereas constitutive expression in C3A cells requires the proximal region of the promoter.

Our results demonstrate that induction of PGLYRP2 mRNA in keratinocytes activated with bacteria has an absolute requirement for the transcription factor NF-κB. The NF-κB family is critical for the regulation of immune responses and includes the proteins p50, p52, p65, and c-Rel, which bind as dimers to the NF-κB consensus sequence (26). Inactive p65 is bound to its inhibitory protein IκB and sequestered in the cytoplasm, and degradation of IκB is essential for the activation of p65 (26, 27). We demonstrate that both NF-κB binding elements, κB1 at −1082 bp and κB2 at −1057 bp, bind the proteins p65 and p50 and both sites are required for the induction of *pglyrp2* transcription in keratinocytes. This is in agreement with the study showing that genes with two κB sites generally require both sites for activity (28). We have shown the requirement for the two κB sites both by the inhibition of degradation of IκB and site-specific mutations within the NF-κB consensus sequences. Despite the absolute requirement for transcription factor NF-κB in the induction of PGLYRP2 mRNA, NF-κB is not sufficient for its expression. HEK293 cells expressing TLR2 or TLR4 activate NF-κB upon exposure to bacteria and express interleukin-8 (21) but not PGLYRP2. Our data are in agreement with the NF-κB-mediated induction of human PGRPs in oral epithelial cells activated by Pam3CSSNA (a synthetic *Escherichia coli*-type triacyl lipopeptide), *E. coli*-type lipid A, diaminopimelic containing desmuramyl peptide, and muramyl dipeptide (29). However, in keratinocytes induction of PGLYRP2 in response to bacteria is not mediated through TLR2 or TLR4, although these receptors are involved in the induction of HBD2 (20, 37, 38). This suggests that the receptors and signal transduction pathways involved in the induction of PGLYRP2 and antimicrobial peptides in keratinocytes are most likely different.

In contrast to keratinocytes, expression of PGLYRP2 mRNA in liver cells is constitutive and the maximal expression of *pglyrp2* in C3A hepatocytes is regulated by sequences between −281 and −157 bp of the promoter. This proximal region of the promoter has several consensus sequences for AP-1. The AP-1 family of transcription factors consists of the Jun and Fos proteins that bind to the AP-1 consensus sequence as dimers and regulate both constitutive and induced expression of genes (39). Jun proteins can bind DNA as homodimers or as heterodimers with Fos proteins. In addition, Jun can also form heterodimers with ATF2, a member of the CREB family of transcription factors. Our data demonstrate that proteins c-Jun and ATF2 bind the AP-1 consensus sequence at approximatelly 260 bp on the *pglyrp2* promoter and this binding site is required for the maximum constitutive expression of PGLYRP2 mRNA in the human liver cell line, C3A. However, a deletion of the AP1 consensus sequence decreased expression of *pglyrp2* by 30%, which indicates that there are other as yet unidentified regulatory element(s) that regulate the high expression of *pglyrp2* in liver cells. Dominant negative ATF2 and c-Jun inhibit expression of the P2 (−607)Luc by about 40 and 30%, respectively. However, these dominant negative proteins show no significant inhibition of the P2 (−260) construct, which is the smallest construct with this AP1 site (data not shown). These results indicate that there may be additional AP1 binding elements within the −607-bp region of the promoter. Inhibition by dominant negative proteins at individual sites may be too small to be measured in our assay, however, inhibition at multiple sites may be cumulative and thus, can be measured. Also, our data indicate that sequences upstream of −281 bp contain repressor elements that may be involved in inhibiting the expression of *pglyrp2*. In the future we will focus on these regions to identify additional regulatory elements involved in the regulation of *pglyrp2* transcription in liver cells.

The amidase activity of PGLYRP2 results in cleavage of the amide bond between MurNAc and L-Ala and thus removes stem peptides from the peptidoglycan molecule. The *in vivo* importance of this activity could be: (i) digestion of peptidoglycan with amidase reduces the cell-activating pro-inflammatory...
activity of polymeric extracellular peptidoglycan (17, 18), which is mediated through TLR2 (40, 41) and would also reduce the recognition of intracellular peptidoglycan fragments, which is mediated by Nod2 and requires muramyl dipeptide (42); (ii) digestion of polymeric peptidoglycan could generate a Nod1-activating peptide, because the minimum structure that activates Nod1 is a tripeptide derived from the stem peptide of a m-diaminopimelic acid-type peptidoglycan (without the glycan) (42); and (iii) PGLYRP2 could have direct antibacterial activity. Therefore, digestion of peptidoglycan with amidase could have a scavenger function to reduce proinflammatory activity of peptidoglycan, it could have a direct antibacterial function, or it could generate Nod1-activating peptides, which would enhance antimicrobial responses.

The induction of PGLYRP2 in human keratinocytes by bacteria and its high constitutive expression in the liver emphasizes the role and importance of the amidase in innate immune responses to bacterial infections. The differential expression may indicate the need for the presence of PGLYRP2 amidase to be constitutive or induced. Human skin is continuously exposed to surface bacteria and other pathogens and may have evolved to tolerate low levels of bacteria and bacterial products such as peptidoglycan. Therefore, PGLYRP2 amidase may be expressed in keratinocytes only in the presence of high numbers of bacteria or in the presence of bacteria that are not part of the normal flora. However, the liver is exposed to blood-borne bacteria and bacterial products, which can quickly lead to a fatal systemic infection, inflammation, and shock. A continuous presence of PGLYRP2 in the serum would effectively and rapidly remove any systemic peptidoglycan, thereby, preventing its proinflammatory effects. Our results identify some of the transcription factors and regulatory elements that are involved in this differential expression of PGLYRP2 amidase.

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