Published in final edited form as:
Glycobiology. 2006 March ; 16(3): 221–229.

Helicobacter Pylori and Toll-Like Receptor Agonists Induce Syndecan-4 Expression in an NF-κB-Dependent Manner

Michael F. Smith Jr., **Jennifer Novotny**, **Virginia S. Carl**, and **Laurey D. Comeau**
University of Virginia Health System, Departments of Medicine, Digestive Health Center of Excellence and Microbiology, Charlottesville, VA USA

Abstract

The syndecans are a family of transmembrane heparan sulfate proteoglycans that have been implicated in a wide variety of biological functions including the regulation of growth factor signaling, adhesion, tumorigenesis, and inflammation. In the current studies we examined the regulation of syndecan-4 gene expression in gastric epithelial cells and macrophages in response to infection with live *Helicobacter pylori* and purified Toll-like receptor (TLR) agonists. *H. pylori*, PAM_3CSK_4 (a TLR2 agonist) and *E. coli* flagellin (a TLR5 agonist) all induced the rapid expression of syndecan-4 mRNA in MKN45 gastric epithelial cells. Similarly, LPS (a TLR4 agonist) also induced the expression of syndecan-4 in macrophages. The *H. pylori*- and TLR-induced increase in syndecan-4 mRNA was blocked by the proteosome inhibitor MG-132 suggesting a role for NF-κB in the regulation of syndecan-4 gene expression. An 895-bp fragment of the human syndecan-4 promoter was cloned upstream of the luciferase reporter. When transfected into MKN45 cells, the activity of this promoter was inducible by *H. pylori* and TLR agonists. Inducible activity of the syndecan-4 promoter was blocked by cotransfection with a dominant negative IκBα expression plasmid. Electrophoretic mobility shift assays (EMSA) demonstrated the presence of a highly conserved NF-κB-binding site. Mutation of this site within the context of the full length syndecan-4 promoter resulted in a complete loss of responsiveness to *H. pylori* and TLR agonists. These results thus demonstrate that the response of the syndecan-4 gene to infectious agents, or their products, is a direct result of NF-κB binding to the promoter and induction of *de novo* transcription.

Keywords

Helicobacter; syndecan; heparan sulfate; Toll-like receptor; NF-KappaB

Abreviations

EMSA, electrophoretic mobility shift assay; FBS, fetal bovine serum; HPRT, hypoxanthine; phosphoribosyltransferase; HSPG, heparan sulfate proteoglycan; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PBS, phosphate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; TLR, Toll-like receptor

Introduction

The Toll-like receptors (TLRs) are a family of “Pattern Recognition Receptors” which recognize conserved microbial products such as lipopolysaccharide (LPS), peptidoglycan (PGN), and non-methylated bacterial DNA (CpG DNA). A common characteristic of the

Address correspondence to: Michael F. Smith, Jr., University of Virginia Health System, PO Box 800708, Charlottesville, VA 22908-0708
Tel : (434)924-2573 ; Fax: (434)243-9645 ; Email:mfs3k@virginia.edu.

agonists recognized by this receptor family is that they are all highly conserved products of microbial metabolism that are vital to the microbes and as such are not generally susceptible to antigenic variability or mutation. The ten different TLRs that have been identified in humans are utilized by innate immune response cells to detect the presence of pathogenic microorganisms. In many cases the microbial agonist has been identified. For example, TLR2 recognizes peptidoglycan (Takeuchi *et al*, 1999) and bacterial lipoproteins (Underhill *et al*, 1999). TLR4 recognizes LPS from most gram negative species (Poltorak *et al*, 1998), TLR5 reacts with flagellin (Hayashi *et al*, 2001), and TLR9 is a receptor for bacterial CpG DNA (Hemmi *et al*, 2000).

The primary function of the TLRs is to alert the immune system to the presence of pathogenic microorganisms. Stimulation of cells via TLR agonists results in the production of numerous immunologically important cytokines, chemokines, and effector molecules. Additionally, microbial products also induce the expression of co-stimulatory molecules on professional antigen presenting cells that are necessary for the activation of T and B cells. Thus, in addition to directly controlling the microbial infection, the innate immune response is also instructive to the adaptive immune response. The expression of Toll-like receptors is not limited to cell types traditionally thought of as innate immune effectors (i.e. monocytes and macrophages). Members of the TLR family can also be found to be expressed in most organs including the lung, heart, and gastrointestinal tract.

Infection with *Helicobacter pylori*, a Gram-negative, microaerophilic, flagellated bacteria that adheres to human gastric mucosa, is strongly associated with gastric ulcers and adenocarcinoma. The specific clinical outcome is determined by the interplay of *H. pylori* virulence factors, host gastric mucosal factors, and the environment. The gastrointestinal epithelium plays critical roles in both the transport of nutrients and as an active barrier against infection. As the first line of defense against the microbe-laden external environment, the epithelial cells lining the gastrointestinal tract must be able sense and respond to potentially pathogenic microorganisms while maintaining tolerance towards the endogenous bacterial flora. Studies from numerous labs have now demonstrated that gastric epithelial cell lines do indeed respond to microbial products through the use of TLRs, and as such can be considered an active part of the innate immune response. Indeed, we have previously demonstrated that live *H. pylori* induced NF-κB activation in MKN45 gastric epithelial cells due to ligation of TLR2 and TLR5, but not TLR4 (Smith, Jr. *et al*, 2003).

The syndecans are a family of four type I transmembrane heparan sulfate proteoglycans (HSPG) which, together with the lipid-linked glypicans, represent the major source of heparan sulfate on the cell surface (Tkachenko *et al*, 2005). The proteins are four distinct gene products which are organized such that the heparan sulfate chains are placed distal to the cell surface and contain a conserved cytoplasmic COOH-terminal with characteristic serine and tyrosine residues. The syndecans are ubiquitously expressed with most cell types expressing at least one, and often multiple, members of the family. Typically, syndecan-1 is the predominant form on epithelial cells, syndecan-2 is on fibroblasts, and syndecan-3 is on neuronal tissue. Syndecan-4 is more ubiquitously expressed.

The syndecans bind to a wide variety of soluble and insoluble extracellular effector molecules such as extracellular matrix components, growth factors, cytokines, and microbial pathogens. Likewise these HSPGs have variably been demonstrated to play important roles in facilitating the formation of active signaling complexes by acting as coreceptors to concentrate and present ligands to the cell surface receptors (Bernfield *et al*, 1999). In some cases the proteins in fact actively participate in the enhancement of cell signaling. For example, syndecan-4 is found as a component of focal adhesions and plays a central role in the activation of protein kinase C,

organization of the actin cytoskeleton, and cell migration (Oh *et al*, 1998;Thodeti *et al*, 2003;VanWinkle *et al*, 2002;Woods *et al*, 2000).

In terms of *H. pylori* infection, several reports in the literature have pointed towards a role for heparan sulfate binding proteins on the bacterial surface as participating in the adhesion of *H. pylori* to cultured cells (Utt & Wadstrom, 1997). Additionally, one report indicates that the vaculating toxin of *H. pylori*, VacA, binds to immobilized heparan sulfate suggesting that HSPGs may play a role in mediating the entry of this toxin into cells (Utt *et al*, 2001) . Because of the suggested role of syndecan-4 as molecule involved in host defense mechanisms, we sought to determine if syndecan-4 expression is regulated in response to microbial-derived factors. The studies described below have indicated that expression of the syndecan-4 gene can be induced in response to infection of gastric epithelial cells with either live *H. pylori* or purified TLR agonists. Furthermore, we have determined that this response is a direct effect of NF-κB binding to a conserved site in the syndecan-4 promoter.

Results

Regulation of syndecan-4 mRNA expression in gastric epithelial cells and macrophages

Previously, we have demonstrated that MKN45 gastric epithelial cells respond to *Helicobacter pylori* through TLR2 and TLR5 (Smith, Jr. *et al*, 2003). In order to better understand the role of Toll-like receptor signaling in gastric epithelial responses we performed a preliminary cDNA microarray experiment using mRNA isolated from MKN45 cells stimulated with either a TLR2 agonist, PAM_3CSK_4, or a TLR5 agonist, *E. coli* flagellin (data not shown). In addition to numerous chemokines, one of the genes found to be upregulated by both stimuli was syndecan-4. Because of the suggested role of syndecan-4 as molecule involved in host defense mechanisms, we sought to determine if syndecan-4 expression is indeed regulated in response to microbial-derived factors.

In order to further explore this response we utilized quantitative RT-PCR to assess the effects of stimulation by PAM_3CSK_4 or FliC on the expression of SDC-4 in MKN45 cells. The results of the representative experiment shown in Figure 1, demonstrated that both the TLR2 agonist (PAM_3CSK_4) and the TLR5 agonist (FliC) induced a time-dependent increase in the expression of SDC-4 mRNA. Over several experiments, we have observed increased levels of SDC-4 mRNA as early as 1 hour following stimulation which peaked at approximately 4–6 hours and declined to near baseline levels by 24–36 hours.

Because *Helicobacter pylori* has been demonstrated to induce chemokine and NF-κB activation through TLR2 and TLR5 (Smith, Jr. *et al*, 2003) the results of the previous experiment suggested that *H. pylori* infection is also likely to induce the expression of syndecan-4. In order to test this hypothesis, MKN45 and AGS cells were infected with live *H. pylori* strain 26695 and mRNA expression assessed by RT-PCR. The results of the representative experiment (of 3) shown in Figure 1B demonstrated that *H. pylori* did indeed induce the expression of SDC-4 mRNA in both gastric epithelial cell lines examined. The MKN45 cells were reproducibly more sensitive to *H. pylori* than the AGS cells. This result would be expected based upon our previous data which demonstrated that AGS cells did not express TLR2 (Smith, Jr. *et al*, 2003). In contrast to the results with the purified TLR2 and TLR5 agonists, the enhanced expression of SDC-4 mRNA observed in response to *H. pylori* infection did not return to baseline for at least 24 hours post-infection. That the observed increases in mRNA expression resulted in increased cell surface expression of syndecan-4 was confirmed by FACS analysis (Figure 2). Using the 8G3 monoclonal antibody we were able to demonstrate that treatment of MKN45 cells with PAM_3CSK_4 (Fig 2A) , FliC (Fig. 2B), or *H. pylori* (Fig. 2C) resulted in an enhancement of cell surface syndecan-4.

Previously, Ishiguro and colleagues demonstrated that intraperitoneal injection of LPS resulted in the up-regulation of syndecan-4 in macrophages and microvascular epithelial cells *in vivo* (Ishiguro *et al*, 2001). Those authors went on to demonstrate that syndecan-deficient mice were more susceptible to LPS-induced shock as a consequence of decreased ability of TGF-β to down-regulate IL-1β production. In order to directly assess the potential effects of microbial products on the expression of syndecan-4 in macrophages, we examined the regulation of syndecan-4 mRNA in response to LPS (a TLR4 agonist) in the murine macrophage cell line RAW 264.7. The results of the representative quantitative RT-PCR experiment shown in Figure 3 demonstrated that LPS rapidly induced syndecan-4 expression in these cells. The kinetics of this response were more rapid than that observed in the gastric epithelial cells with mRNA levels reaching maximum at 3–4 hours post-stimulation and returning to baseline levels within 8 hours. Similar results were observed using primary bone marrow-derived macrophages (data not shown). Taken together, these results demonstrated that syndecan-4 expression is rapidly induced by live bacteria or bacterial products in epithelial cells and macrophages, two cell types which comprise the first line of defense in the innate immune response.

NF-κ B-dependent regulation of syndecan-4

One hallmark of many of the genes which are rapidly induced through Toll-like receptors is that they are regulated at least in part via NF-κB. Previously Zhang, et al. demonstrated that the TNF-α-induced syndecan-4 expression in a human umbilical vein endothelial cell line could be decreased by pretreatment with the proteosome inhibitor lactacystin suggesting a role for NF-κB in this response (Zhang *et al*, 1999). Likewise, Zhou et al. demonstrated that siRNA blockade of NF-κB p65 expression also inhibited TNF-α-dependent increases in syndecan-4 expression in HeLa cells (Zhou *et al*, 2003). In order to determine if NF-κB was also essential for the increase in syndecan-4 expression observed in response to bacterial products, the experiments shown in Figure 4 were performed. In Figure 4A MKN45 cells were pretreated with the proteosome inhibitor, MG-132, prior to infection with *H. pylori* for 6 hours. Similarly, in figure 4B, MKN45 cells were treated with the proteosome inhibitor prior to stimulation with PAM_3CSK_4 or FliC for 6 hours. The expression of syndecan-4 mRNA was analyzed by quantitative RT-PCR. In both cases, MG-132 treatment resulted in a near complete inhibition of syndecan-4 gene expression. These results therefore indicate that microbial-induced syndecan-4 expression in MKN45 cells is dependent upon NF-κB activation.

Characterization of a functional NF-κ B binding site in the human syndecan-4 promoter

We next wanted to determine if the microbial-induced expression of syndecan-4 was in fact due to increased transcription of the syndecan-4 gene and if this response was dependent upon NF-κB. Using the BAC clone RP11-358A17 as a template, we PCR amplified a 935-bp fragment of the human syndecan-4 gene corresponding to sequences from −895 to +40, relative to the transcription start site (+1). This fragment was cloned upstream of the luciferase reporter gene. This reporter construct was transiently co-transfected into MKN45 cells with or without a dominant negative IκB expression plasmid, pCMV-IκB S32/36A (Brockman *et al*, 1995). This dnIκB construct expresses a mutant IκBα protein which cannot be phosphorylated at serines 32 and 36 and thus renders it insensitive to degradation. The resultant IκB/NF-κB complex is thereby maintained in the cytoplasm. As shown in Figure 5, *H.pylori*, infection induced a significant (6 to 7-fold) increase in syndecan-4 promoter activity which was nearly completely blocked by co-transfection of IκB-S32/36A. The same effect of IkB S32/36A overexpression was observed on syndecan-4 promoter activity induced in response to PAM_3CSK_4 or FliC (data not shown). Previously, Zhang et al. demonstrated that a 690-bp promoter fragment could generate a 2-fold response to TNF-α in an endothelial cell line (Zhang *et al*, 1999). These results therefore indicated that the syndecan-4 gene is transcriptionally upregulated in response to *H. pylori* infection and that response is dependent upon NF-κB.

While the results described above and in the literature(Zhou *et al*, 2003;Zhang *et al*, 1999) are consistent with a role for NF-κB in the regulation of syndecan-4 gene expression, neither of the previously published studies formally demonstrated NF-κB binding sites within the promoter of either the mouse or human genes. Both the mouse and the human syndecan-4 genes contain identical potential NF-κB binding sites (GGGGAATTCC) upstream of the transcription start site: −97 to −88 in human and −84 to −75 in mouse. In order to determine if the region of the human syndecan-4 promoter between −97 and −88 is in fact a *bona fide* NF-κB binding site we performed a series of EMSA experiments with nuclear extracts from MKN45 cells. EMSA was performed using a radiolabeled double-stranded oligonucleotide corresponding to sequences of the human syndecan-4 promoter between −112 and −77. In the representative experiment shown in Figure 6A, MKN45 cells were stimulated with live *H. pylori* for 60 or 120 minutes, prior to isolation of nuclear proteins. This EMSA demonstrated the presence of an H. pylori-inducible complex which was evident 1h after infection. To confirm that this complex did indeed contain NF-κB proteins, the antibodies specific for two components of the NF-κB complex, p50 and p65, or PU.1 (as a negative control) were added to nuclear extracts from cells infected with *H. pylori* for 60 minutes prior to the addition of radiolabeled probe. Addition of either of the two antibodies specific for NF-κB proteins, but not PU.1 decreased the formation of the inducible complex thus indicating that the *H. pylori*-inducible complex contained NF-κB p50 and p65. We also examined the ability of stimulation specifically via TLR2 or TLR5 to induce the formation of this complex. In Figure 6B and 6C, EMSAs were performed using nuclear extracts prepared from MKN45 cells stimulated with PAM_3CSK_4 or FliC for 30 or 60 minutes. Results of these experiments also demonstrated that the purified TLR agonists could induce NF-κB binding to the syndecan-4 promoter fragment. Furthermore, as shown in the right side of Figure 6B, this complex could be competed using unlabeled oligonucleotides corresponding to the NF-κB site from the Igκ enhancer or the WT syndecan-4 sequence but not by oligonucleotides containing a 6 base-pair mutation within the syndecan-4 NF-κB site. Taken together, these EMSA studies therefore demonstrated that the human syndecan-4 gene contains a *bona fide* NF-κB binding motif.

Finally, in order to determine if this NF-κB binding site was indeed functional and involved in the response of the human syndecan-4 gene to *H. pylori* and TLR agonists we mutated the NF-κB site in the human syndecan-4 promoter reporter construct to GGctcgagCC. This is the same sequence which was demonstrated in Figure 6B to be incapable of binding NF-κB. Both the wild type and mκB reporter constructs were then transiently transfected into MKN45 cells. As shown in Figure 7A, mutation of the NF-κB site resulted in a nearly complete inhibition of inducible promoter activity in responses to *H. pylori* infection. Likewise the mutant promoter construct could not be activated in response to stimulation with PAM_3CSK_4 or flagellin (Figure 7B). These data therefore demonstrated that DNA sequences, consistent with an NF-κB binding site, within the human syndecan-4 promoter were essential for the ability of the gene to respond to bacteria and bacterial products. Notably, mutation of this site had no effect on basal (unstimulated) promoter activity demonstrating a specific role for this NF-κB-binding site in inducible responses

Disscussion

Several studies have indicated pivotal roles for the syndecans in host defense(Ishiguro *et al*, 2002). Studies from the lab of Merton Bernfield, have demonstrated that *Pseudomonas aeruginosa* through its virulence factor LasA, induces the *in vitro* shedding of syndecan-1 from epithelial cells (Park *et al*, 2001). Surprisingly, syndecan-1 deficient mice are more resistant to *P. aeruginosa* infection but become more susceptible when given purified syndecan-1 ectodomains indicating that *P. aeruginosa* exploits the shedding of syndecan-1 ectodomains as a virulence factor. On the other hand, studies with syndecan-4 knockout mice have indicated that they are in fact more susceptible to LPS-induced lethality (Ishiguro *et al*, 2001). These

and other studies in the literature point to a potential role for the syndecans in the control of host defense against pathogenic microbes.

The current study examined the expression of syndecan-4 in the gastric epithelial cell line MKN45 in response to infection with *H. pylori* or stimulation with purified TLR agonists. We observed that the expression of the syndecan-4 mRNA was significantly increased in response to live *H. pylori*, PAM_3CSK_4, and flagellin. Furthermore, as suggested by Ishiguro et al. (Ishiguro *et al*, 2001), syndecan-4 mRNA expression was also increased in response to LPS treatment of the murine macrophage cell line RAW264.7. These findings suggest that syndecan-4 may play a significant role in the host defense against microbial infection. A role for LPS-induced syndecan-4 expression in macrophages has been suggested (Ishiguro *et al*, 2001). The increased sensitivity of syndecan-4-deficient mice to endotoxic shock was likely due to a decreased ability of their macrophages to downregulate the expression of IL-1β in response to TGF-β, a ligand for syndecan-4. This would imply that sydecan-4 plays a negative regulatory role in the host immune response.

The role which syndecan-4 might play in the epithelial cell response to microbial infection remains open to speculation. Several different mechanisms can be envisioned. Like a number of other cell surface receptors, the syndecan ectodomains are shed from the cell surface both constitutively during the course of heparan sulfate turnover and in a regulated fashion in response to tissue injury, growth factor stimulation, and microbial infection (Fitzgerald *et al*, 2000;Subramanian *et al*, 1997). These shed ectodomains maintain their abilities to interact with target ligands and alter the ability of these extracellular effectors to induce cellular responses. Many pathogens, including *H. pylori*, may exploit cell surface heparan sulfate proteoglycans (HSPGs) in order to bind host cells (Utt *et al*, 2001;Utt & Wadstrom, 1997;Duensing *et al*, 1999). Thus induced shedding of syndecan-4 ectodomains may play a protective role by decreasing *H. pylori* binding to the cell surface. In fact, a number of studies have demonstrated the increased production of heparin-binding EGF (HB-EGF) in *H. pylori*-infected tissues (Romano *et al*, 1998;Schiemann *et al*, 2002;Tuccillo *et al*, 2002) which could potentially induce the shedding of syndecans-4 from the cell surface (Fitzgerald *et al*, 2000). The physiological role of syndecan-4 in *H. pylori* infection is currently under investigation in our lab.

A second possible role for increased syndecan-4 expression during an inflammatory response may relate to the described abilities of HSPGs, including the syndecans, to bind a variety of host-derived growth factors and chemokines (Tkachenko *et al*, 2005;Slimani *et al*, 2003). It has been proposed that cell surface heparan sulfates can bind leukocyte chemoattractants and promote leukocyte recruitment through the formation of cell surface chemokine gradients (Gotte, 2003;Gotte & Echtermeyer, 2003;Lipscombe *et al*, 1998;Kohrgruber *et al*, 2004). Since the primary response of epithelial cells to microbial infection is the production of numerous chemokines, it could be postulated that increases in cell surface syndecan-4 expression may facilitate the development of chemokine gradients. The establishment of such chemokine gradients is essential for the recruitment of effector leukocytes into the area of infection. Indeed, macrophage and neutrophil recruitment is one of the hallmarks of the *H. pylori*-induced inflammatory response. Alternatively, as described by Ishiguro et al. syndecan-4 may play a negative feedback role by aiding in the binding of anti-inflammatory molecules (Ishiguro *et al*, 2001). Additionally, HSPGs have been demonstrated to bind the important anti-inflammatory cytokine IL-10 and modulate its activity (Salek-Ardakani *et al*, 2000). Thus, this ability to bind critical effector molecules has the potential to result in positive or negative effects on the host response to infection.

The other significant findings from the current study are the demonstration that microbial-induced syndecan-4 expression is mediated via the activation of NF-κB and the formal identification of a functional NF-κB-binding site within the human syndecan-4 promoter.

Previous studies by Zhang et al. (Zhang *et al*, 1999) and Zhou et al. (Zhou *et al*, 2003) have demonstrated an important role for NF-κB activation in the inducible expression of syndecan-4 by TNF-α. However, neither study specifically determined if NF-κB acts directly to induce syndecan-4 expression or if the effect on syndecan-4 expression was secondary to the inhibition of NF-κB activity. In the current study, we demonstrated that the microbial-induced expression of syndecan-4 is a direct result of NF-κB binding to a conserved site in the proximal promoter region of the gene. By EMSA we demonstrated that *H. pylori*, PAM_3CSK_4, and flagellin could all induce the binding of NF-κB to an oligonucleotide derived from the human syndecan-4 promoter. More importantly, mutation of that NF-κB binding site resulted in a loss of responsiveness of the syndecan-4 promoter to all three stimuli. Taken together, these results provide the first formal demonstration that NF-κB is directly involved in the regulation of syndecan-4 gene expression. Furthermore, the kinetics of syndecan-4 mRNA induction following microbial stimulation, i.e. as early as 1 hour, suggests that this is a primary response to infection.

In summary, *H. pylori* or other related microbial products, can induce the expression of syndecan-4 mRNA in gastric epithelial cells and macrophages. The response of the syndecan-4 gene to infection is a direct result of the ability of these microbes and their products to activate NF-κB via interactions with members of the Toll-like receptor family. Activated NF-κB can then bind directly to a conserved site on the syndecan-4 promoter to enhance transcription of the gene. This response may play an important role in the development of the host response to infection which needs to be investigated further.

Experimental Procedures

Cell culture and transfection

MKN45 cells were purchased from the JCRB Cell Bank (Japan), AGS and RAW 264.7 cells were from ATCC. AGS cells were cultured in HAMs F-12 +10 % FBS and MKN45 in RPMI 1640 + 10 % FBS (Hyclone). MKN45 cells were transfected in 24 well plates using LipofectAmine 2000 (Invitrogen). Each transfection contained 600 ng syndecan-4/Luc, 50 ng pTK-renilla (Promega) and 1.5 μl Lipofectamine 2000. Transfections were performed in triplicate, cultured for 24 h, and then stimulated as indicated. Luciferase activities were determined using the dual luciferase kit from Promega and all activities normalized to the activity of the cotransfected TK-renilla plasmid. Transfections with the dominant negative S32/36A IκB plasmid contained, in addition to the luciferase plasmids, 100 ng of either the dnIκB plasmid or the empty pCMV4 vector as a negative control. The S32/36A IκBα plasmid was a gift of Dean Ballard, Vanderbilt University.

TLR agonists PAM_3CSK_4 was purchased from EMC Microcollections (Tuebingen, Germany). Recombinant His-tagged *E. coli* flagellin was prepared as previously described by Donnelly and Steiner (Donnelly & Steiner, 2002). The *E. coli FliC* expression plasmid was a gift from Ted Steiner, Univ. of British Columbia, Vancouver, CA. Following nickel agarose affinity purification, the protein was concentrated, imidazole removed and buffer exchanged into phosphate buffered saline (PBS) using an Amicon Ultra centrifugal filter device (Millipore, Bedford, MA). Finally, endotoxin was removed by chromatography on polymyxin B agarose (Pierce). Purity and protein concentration was determined by SDS-PAGE followed by coomassie blue staining. Bovine serum albumin was used as a known concentration standard. FliC prepared in this way was devoid of TLR2 or TLR4 agonist activity as determined using HEK293 cells transfected with TLR2 or TLR4 as previously described (Carl *et al*, 2002). *Helicobacter pylori*, strain 26695, was from K. Eaton (Ohio State Univ. Columbus, OH) and was cultured as previously described (Smith, Jr. *et al*, 2003).

FACS analysis of syndecan-4 expression

MKN45 cells were stimulated as indicated and removed from the plates by gentle scraping in PBS+2 mM EDTA and stained for FACS analysis essentially as described by Freissler, et al (Freissler *et al*, 2000). Cells were incubated with mouse monoclonal 8G3 (a kind gift from G. David, University of Leuven, Belgium) (David *et al*, 1992) at 30 μg/ml for 1h in PBS with 2% FBS, 1mM $MgCl_2$, 0.5 mM $CaCl_2$, washed twice, and then incubated with FITC-conjugated goat anti-mouse antibody (Santa Cruz). Isotype matched non-specific mouse monoclonal was used to assess background staining. Dead cells were excluded based upon staining with 7-amino-actinomycin D.

Cloning of the human syndecan-4 promoter

A bacterial artificial chromosome containing the entire human syndecan-4 gene, RP11-358A17, was obtained from the BACPAC Resources Center at the Children's Hospital Oakland Research Institute (Oakland, CA). A 935-bp fragment of the human syndecan-4 gene was amplified using pfx polymerase (Invitrogen) from the BAC clone using hSDC4F3 (5′-GCATAAGCTTCACCTCTCTGGCTCAAGCAGTCCT-3′) and hSDC4 +40R (5′-CGATAAGCTTGGCACCGCGGACTGGAGAAG-3′). The fragment was digested with *HindIII* and cloned upstream of the luciferase gene in pA_3Luc (Maxwell *et al*, 1989). The sequence of the cloned promoter was verified by automated DNA sequencing. To generate the promoter containing the mutated NF-κB site, recombinant PCR was performed using the cloned wild type promoter was used as a template. To generate a mutation in the putative NF-κB binding element, primers hSDC4 mκB F (5′-GGCCTCGCTTCCACTGGCTCGAGCCGGGCGGGGTG-3′) and hSDC4 mκB R(the reverse complement of A) were used to mutate six base pairs within the putative NF-κB, thus creating a new *XhoI* site. PCR reaction 1 was performed with hSDC4 F3 and hSDC4 mκBR. A second reaction was performed with hSDC4 mκB F and hSDC4 +40R. The resulting PCR products were purified and equal molar amounts mixed and used as templates in a third PCR reaction using the two outside primers (hSDC4 F3 and hSDC4 +40R). The final PCR product was digested with *HindIII* and cloned into pA_3Luc.

Electrophoretic mobility shift assays (EMSA)

Nulcear extracts were prepared and EMSA reactions were performed essentially as previously described (Smith, Jr. *et al*, 98 A.D.). Sequences of the oligonucleotides used are shown in Table I. Antibodies specific for NF-κB p50 (SC-114), p65 (SC-109), and PU.1 (SC-352) were obtained from Santa Cruz and used at 2 μl per reaction. Following electrophoresis, the gels were dried and imaged using A Molecular Dynamics Storm 840 phosphorimager.

Quantitative RT-PCR

Total RNA was purified using the Trizol reagent (Invitrogen). Reverse-transcription (RT) of 0.5 μg of total cellular RNA was performed in a final volume of 20 μl containing 5X first strand buffer, 1 mM of each dNTP, 20 U of placental RNase inhibitor, 5 μM of random hexamer, and 9U of MMLV reverse transcriptase (Invitrogen). After incubation at 37°C for 45 min, the samples were heated for 5 min at 92°C to end the reaction and stored at −20°C until PCR use. Two μl of cDNA was subjected to real-time, quantitative PCR using the MJ Research Opticon system with SYBR Green I (Molecular Probes) as a fluorescent reporter. Syndecan-4 and HPRT cDNAs were amplified in separate reactions. Duplicate PCR reactions were performed for each sample and the average threshold cycle number was determined using the Opticon software. Levels of syndecan-4 expression were normalized to HPRT levels using the formula $2^{(Rt-Et)}$ where Rt is the threshold cycle for the reference gene (HPRT) and Et is the threshold cycle for the experimental gene ($\Delta\Delta C_T$ method). Data are thus expressed as arbitrary units. Sequences for the oligonucleotides used are provided in Table I.

Acknowledgements

This work was supported by NIH RO1-AI34358 and American Cancer Society RSG-01-034-01-TBE (MFS)

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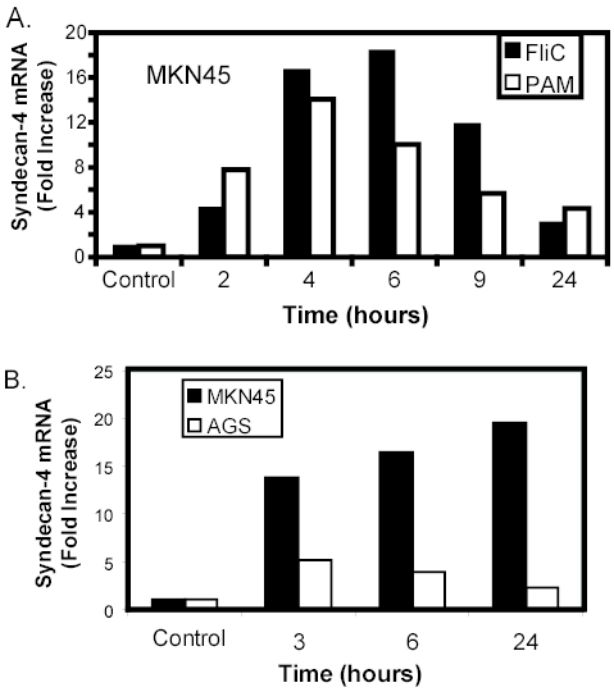

Figure 1. Regulation of syndecan-4 mRNA expression in gastric epithelial cells

A. MKN45 cells were stimulated with either 100 ng/ml PAM_3CSK_4 or 100 ng/ml recombinant FliC for the indicated time prior to isolation of total mRNA. Expression of mRNA for syndecan-4 was determined by quantitative RT-PCR as described in materials and methods. B. MKN45 or AGS cultures were infected with live *H. pylori* (MOI 100:1) for the indicated time prior to isolation of total mRNA and analysis of syndecan-4 mRNA expression. Similar results were observed in two additional experiments.

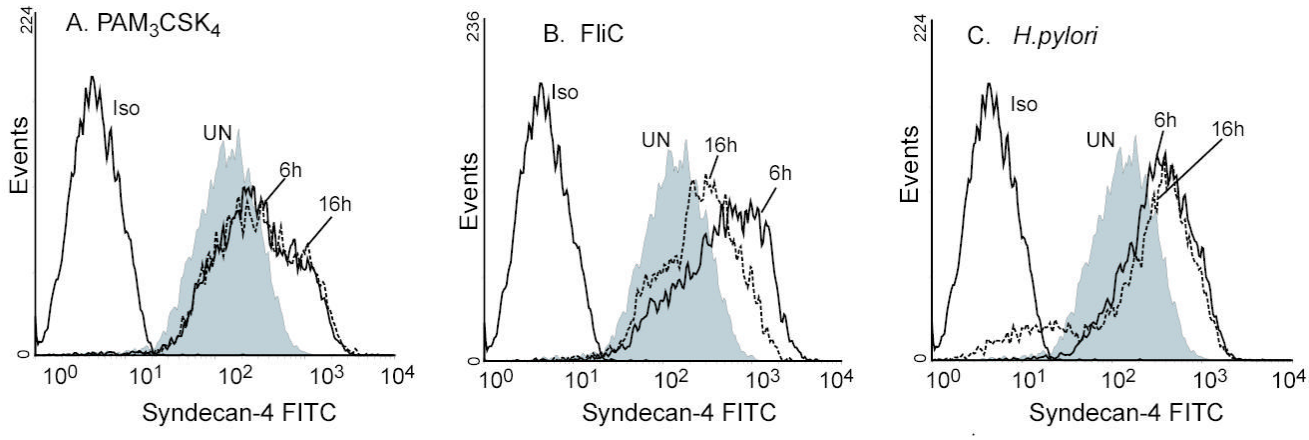

Figure 2. Inducible cell surface expression of syndecan-4 on MKN45 cells

MKN45 cells were stimulated for 6 or 16 hours with PAM_3CSK_4 (A), FliC (B), or live *H. pylori;*MOI 50:1 (C). Iso: isotype control; UN: Unstimulated (shaded); 6 h (solid line); 16 h (dashed line). Similar results were obtained in at least 2 separate experiments with each stimulus.

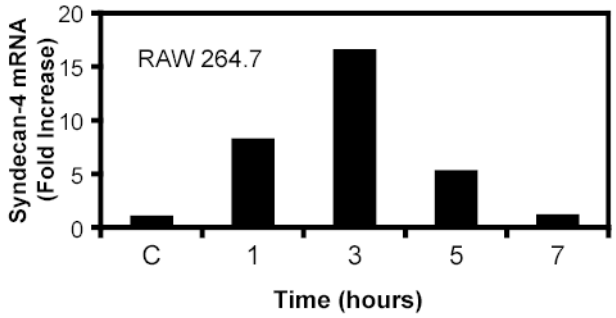

Figure 3. LPS-induced expression of syndecan-4 in RAW264.7 macrophages

RAW264.7 cells were stimulated with 1 μg/ml LPS for the indicated time prior to isolation of total mRNA and analysis of syndecan-4 mRNA expression as in Figure 1. Similar results were observed in two additional experiments

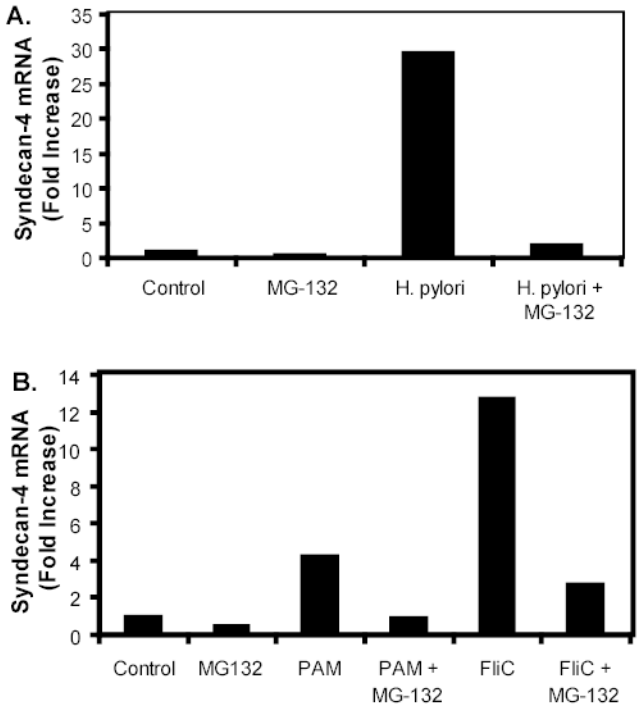

Figure 4. Inhibition of syndecan-4 mRNA expression by MG-132

MKN45 cells were pretreated for 30 min with either DMSO control or 5 μM MG-132 prior to stimulation by live *H. pylori* (A) or 100 ng/ml PAM_3CSK_4 or 100 ng/ml FliC (B) for 3 hrs. Syndecan-4 mRNA expression was determined as described in Material and Methods. Similar results were observed in two additional experiments.

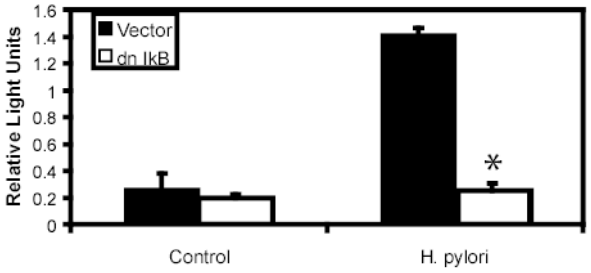

Figure 5. *H. pylori*-induced syndecan-4 promoter activity is NF-κB dependent

MKN45 cells were transiently transfected with SDC-4Luc, phRLTK, and either empty pCMV4 or pCMV4-IκB S32/36A (dnIκB). Thirty-six hours after transfection cultures were infected with *H. pylori*. Luciferase activities were determined 6h later and normalized to cotransfected pRLTK activity. Results are means +/− std. dev. of triplicate transfections and is a representative experiment of three performed. * $p<0.05$ by two-tailed paired t test compared to empty vector.

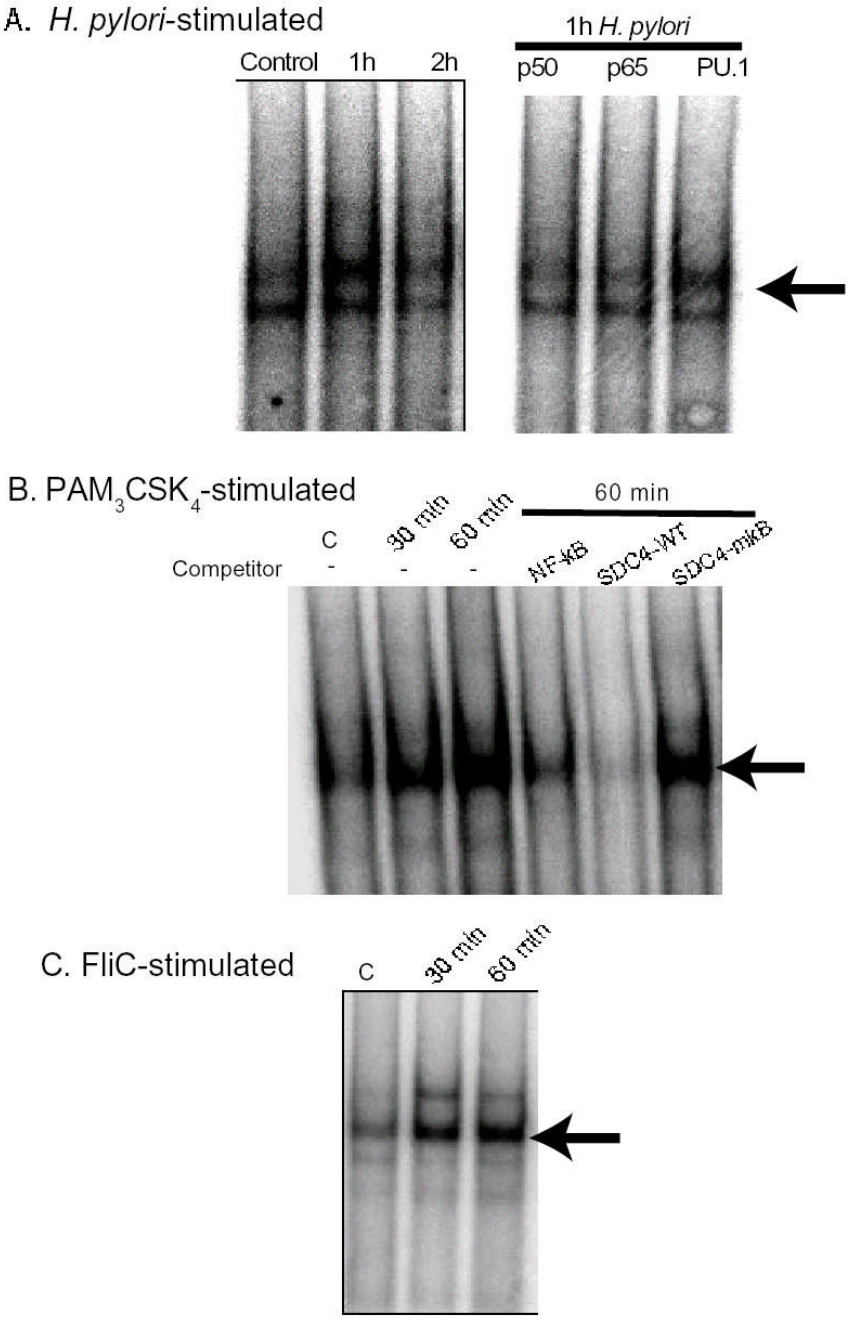

Figure 6. Electrophoretic mobility shift assays (EMSA) of an NF-κB-binding site in the human syndecan-4 promoter

Nuclear extracts were prepared from MKN45 cells stimulated as indicated and reacted with a ^{32}P-labeled oligonucleotide corresponding to the NF-κB-like site in the human syndecans-4 gene. A: *H. pylori*-infected cells. Left: time course Right: Extracts from cells infected for 1h were preincubated with antibodies specific for NF-κB p50, p65, or PU.1 prior to incubation with the radiolabeled oligonucleotide. B: PAM_3CSK_4-stimulated cells. Extracts from cells stimulated for 60 min were incubated with 10-fold molar excess of the indicated unlabeled competitor oligonucleotide prior to the addition of the radiolabeled probe. C: FliC-stimulated cells. All results are representative of three experiments.

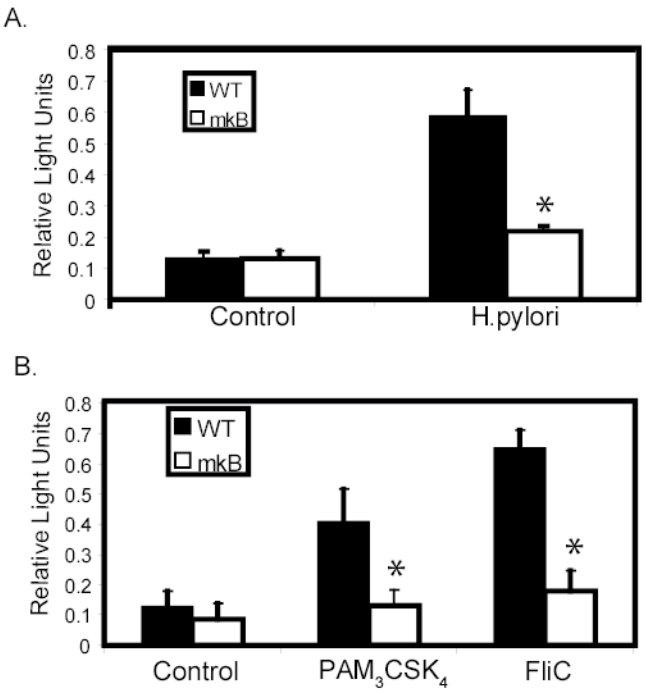

Figure 7. Mutation of the syndecan-4 NF-κB-binding site inhibits inducible promoter activity

MKN45 cells were transiently transfected with either SDC-4Luc (WT) or SDC-4mκBLuc (mκB) and stimulated for 6 h with *H. pylori* (A) or PAM_3CSK_4 or FliC (B) prior to assay for luciferase activity as described above. Results are means +/− std. dev. of triplicate transfections and is a representative experiment of three performed. * $p<0.05$ by two-tailed paired t test compared to wild type.

Table I

Oligonucleotide Sequences

Gene	Forward Primer	Reverse Primer	Product Size (bps)	Accession Number
hSDC-4	CCGGAGCCCTACCAGACGAT	AGGCACCAAGGGATGGACAA	137	BC030805
mSDC-4	GCCCAGGGCAGCAACATCT	CATGCGGTACACCAGCAGCA	114	BC005679
hHPRT	TTGGAAAGGGTGTTTATTCCTCA	TCCAGCAGGTCAGCAAAGAA	146	M31642
mHPRT	TGCCGAGGATTTGGAAAAAGTG	CACAGAGGGCCACAATGTGATG	116	NM_013556
SDC4 NF-κ B	GGCCTCGCTTCCACTGGGGAATTCCGGGCGGGGTG	CACCCCGCCCGGAATTCCCCAGTGGAAGCGAGG		
SDC4 mκ B	GGCCTCGCTTCCACTGGCTCGAGCCGGGCGGGGTG	CACCCCGCCCGGCTCGAGCCTGTGGAAGCGAGGCC		
Igκ B	CTCGGGACTTTCCGAGCT	AGCTCGGAAAGTCCCGAG		