Cell, Vol. 91, 605–615, November 28, 1997, Copyright ©1997 by Cell Press

Acidic Sphingomyelinase Mediates Entry of N. gonorrhoeae into Nonphagocytic Cells

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Summary

Invasion of human mucosal cells by N. gonorrhoeae via the binding to heparansulfate proteoglycan receptors is considered a crucial event of the infection. Using different human epithelial cells and primary fibroblasts, we show here an activation of the phosphatidylcholine-specific phospholipase C (PC-PLC) and acidic sphingomyelinase (ASM) by N. gonorrhoeae, resulting in the release of diacylglycerol and ceramide. Genetic and/or pharmacological blockade of ASM and PC-PLC cause inhibition of cellular invasion by N. gonorrhoeae. Complementation of ASM-deficient fibroblasts from Niemann-Pick disease patients restored N. gonorrhoeae–induced signaling and entry processes. The activation of PC-PLC and ASM, therefore, is an essential requirement for the entry of N. gonorrhoeae into distinct nonphagocytic human cell types including several epithelial cells and primary fibroblasts.

Introduction

Entry of Neisseria gonorrhoeae (Ngo) into human mucosal epithelial cells is considered a crucial event during infection (McGee et al., 1983; Apicella et al., 1996). The first contact is established by bacterial pili proteins (Virji and Heckels, 1984; Rudel et al., 1995) followed by tight adherence via the phase variable colony opacity-associated (Opa) proteins (Lambden et al., 1979; Swanson et al., 1988, Makino et al., 1991). Gonococci are able to express about 11 different Opa proteins (Stern et al., 1986; Bhat et al., 1991; Kupsch et al., 1993), but usually only one particular Opa protein of a strain confers invasion of epithelial cells (Makino et al., 1991). Likewise, Opa_{30} and $Opa_{27.5}$ represent epithelial cell invasion-associated adhesins of the Ngo strains MS11 and VP1, respectively (Makino et al., 1991), binding heparansulfate proteoglycan (HSPG) receptors of the syndecan family (Chen et al., 1995; van Putten and Paul, 1995), and are crucial components of an actin filament–dependent entry process (Bessen and Gotschlich, 1986; Makino et al., 1991; Grassmé et al., 1996). Other Opa proteins (Opa_{52} and Opa_{60}) permit interaction with phagocytic cells (Kupsch et al., 1993) via glycoproteins of the CD66 family and thereby promote an opsonin-independent bacterial uptake by professional phagocytes (Chen and Gotschlich, 1996; Virji et al., 1996; Bos et al., 1997; Gray-Owen et al., 1997). In addition to the Opa proteins, several other proteinacious gonococcal factors (Fussenegger et al., 1996), including the major outer membrane porins PorB (i.e., P1.A and P1.B) (Gotschlich et al., 1987; Weel et al., 1991; Rudel et al., 1996) and the phase-variable lipopolysaccharide (LPS) (van Putten, 1993), have been implicated in the invasion process. An Ngo-expressed glycolipid-specific adhesin has also been identified to interact with target cells, but its role in epithelial cell invasion has not been elucidated (Paruchuri et al., 1990). Finally, the binding of vitronectin to HSPG-specific Opa proteins enhances the entry process of Ngo into certain human cell lines including HeLa cervical or Chinese hamster ovary cells but not Chang conjunctiva cells (Duensing and van Putten, 1997; Gómez-Duarte et al., 1997).

In the present study, we aimed to identify cellular mechanisms involved in the Opa-mediated HSPG-dependent entry of Ngo into nonphagocytic human cells. We provide evidence that a signaling pathway involving stimulation of the phosphatidylcholine-specific phospholipase C (PC-PLC) and acidic sphingomyelinase (ASM) is critical for gonococcal entry into several human cell types including Chang or RT112 epithelial cell lines and primary fibroblasts. Inhibition of either PC-PLC by D609 or ASM by imipramine prevents invasion of Ngo. In addition, ASM-deficient fibroblasts obtained from Niemann-Pick disease type A (NPDA) patients do not internalize Opa-expressing HSPG-specific gonococci. Transfection with ASM restores gonococcal uptake by these ASM-deficient host cells. The data suggest a crucial role of PC-PLC and ASM in the uptake of Ngo by several nonphagocytic mucosal cell types and provide evidence for a novel function of the ASM-signaling pathway.

Results

PC-PLC Activation Is Required for Internalization of N. gonorrhoeae by Human Chang Conjunctiva Epithelial Cells

To identify signaling molecules involved in Ngo uptake by epithelial cells, several inhibitors of lipid metabolism

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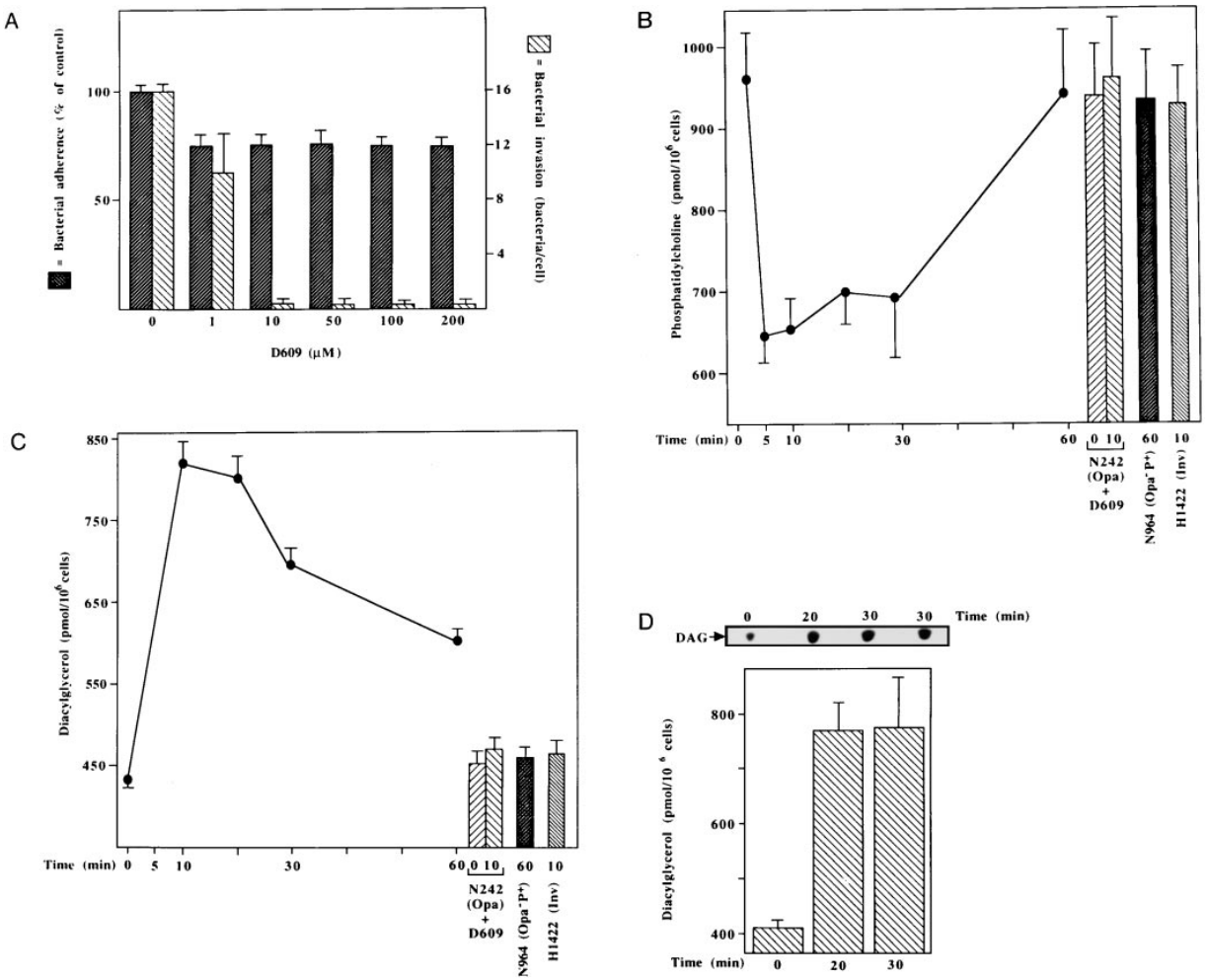

Figure 1. N. gonorrhoeae Invasion into Epithelial Cells Correlates with an Activation of PC-PLC

(A) Invasion of Chang cells by Ngo N242 $Opa_{27.5}$ is dose dependently prevented by the PC-PLC inhibitor D609 (15 min preincubation), whereas adhesion of bacteria is only slightly affected. Bacterial invasion and adherence were determined by crystal violet assays. Shown is the mean ± SD of three independent experiments.

(B–D) The invasion of N242 $Opa_{27.5}$ into Chang cells correlates with a rapid consumption of PC (B) and a release of DAG (C and D), which are inhibited by preincubation with the PC-PLC inhibitor D609 (B and C). The noninvasive but adherent gonococcal strain N964 $Opa^- P^+$ (60 min infection time) or H1422 Inv^+ did not induce these changes, showing the specificity of PC-PLC activation for invasive Ngo.

PC was isolated from [^{3}H]choline chloride–labeled Chang cells by organic extraction (B). DAG release was determined by activation of a DAG-sensitive E. coli kinase in the presence of [γ-^{32}P]ATP (C) or directly measured by extraction from [^{14}C]lyso-phosphatidylcholine-labeled cells (D). All lipids were analyzed by TLC separation and LSC.

were tested. These studies (Figure 1A) revealed an almost complete inhibition of Ngo N242 $Opa_{27.5}$ uptake by D609, a drug that inhibits PC-PLC (Sauer et al., 1984; Schütze et al., 1992). D609 was added to Chang cells 15 min prior to infection with N242 $Opa_{27.5}$ and did not affect the viability of bacteria or target cells judged by survival assays and microscopic examination. Inhibition of Ngo internalization by D609 was dose-dependent and almost complete at a concentration of 10 μM, whereas bacterial adherence was reduced by only 25%–30% (Figure 1A). The inhibitory effect of D609 on Ngo internalization was reversible after removal of the drug, indicating that D609 did not irreversibly affect the ability of the bacteria to invade epithelial cells. Invasion or adherence of H1422 Inv^+, an Escherichia coli strain expressing the Yersinia pseudotuberculosis invasin that binds to integrins and thereby mediates activation of Src-like tyrosine kinases (Isberg et al., 1987; Isberg and Leong, 1990; Rosenshine et al., 1992a), was not affected by D609 at concentrations up to 100 μM (data not shown).

We therefore tested whether internalization of Ngo by Chang cells induces changes in lipid metabolism. Infection of Chang cells with N242 $Opa_{27.5}$ resulted in a strong and rapid consumption of PC (Figure 1B), correlating with a release of diacylglycerol (DAG), which was inhibited by preincubation of target cells with D609 (Figures 1B and 1C). These changes were specific for Ngo, since they were not triggered by H1422 Inv^+ (Figures 1B and 1C). The DAG kinase used in the assay was not influenced by a gonococcal or mammalian factor, which might be triggered in the cells upon bacterial infection, since the addition of increasing amounts of DAG or C_{16}–ceramide resulted in a proportional increase in ^{32}P incorporation (data not shown). An activation of PC-PLC by simple adherence of the bacteria to epithelial cells was excluded, since exposure of Chang cells to Ngo

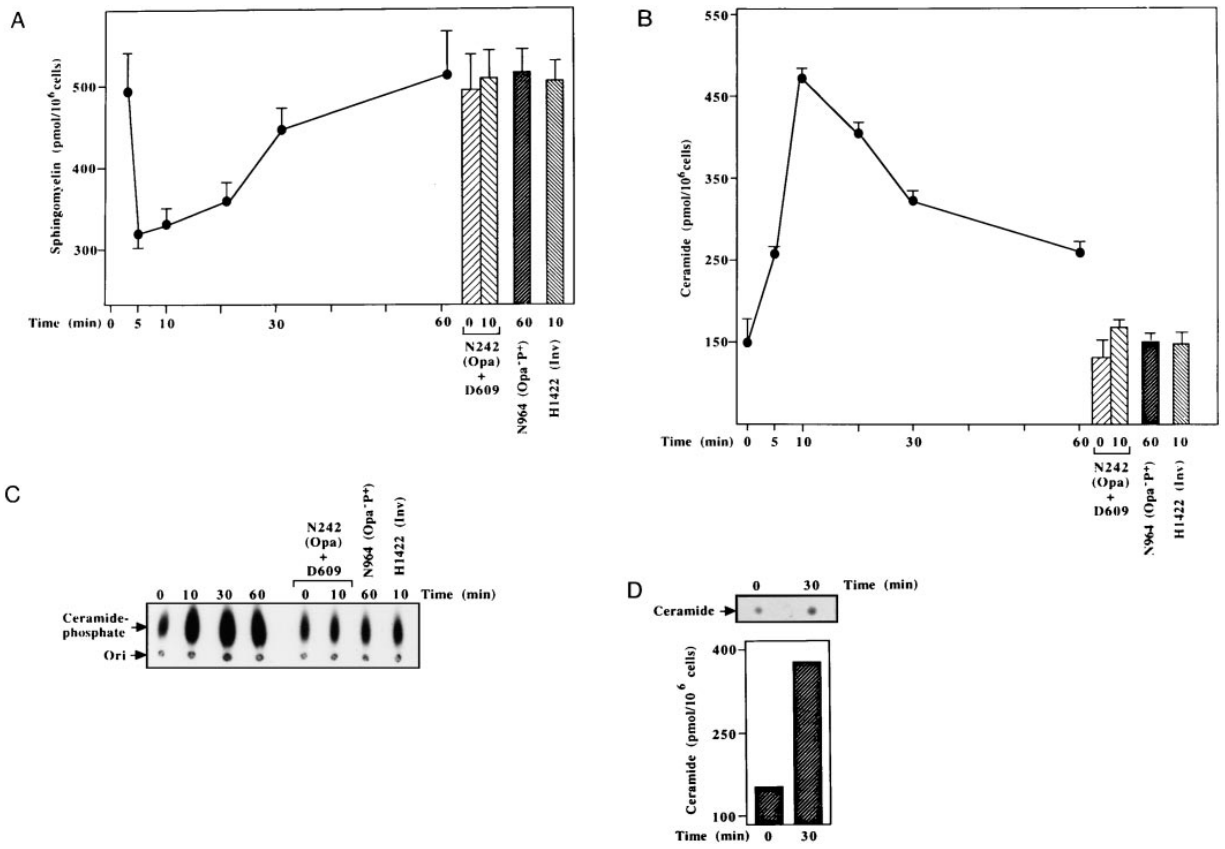

Figure 2. Invasion of N. gonorrhoeae Induces a Consumption of Sphingomyelin and a Release of Ceramide

Uptake of Ngo by Chang cells correlates with a rapid consumption of SM (A) and a release of ceramide (B–D), which are inhibited after 15 min preincubation with D609 (A–C). The noninvasive gonococcal strain N964 Opa$^-$ P$^+$ or H1422 Inv$^+$ did not induce any cellular SM consumption or release of ceramide (A–C). SM consumption was determined by organic extraction of [^{3}H]choline chloride–labeled cells after infection. Ceramide release was determined by incubation of organic cell extracts with a ceramide-sensitive E. coli kinase in the presence of [γ-^{32}P]ATP (B and C). Alternatively, ceramide was directly determined by extraction from [^{3}H]serine-labeled cells (D). Lipids were separated by TLC and analyzed by LSC (A and B) or autoradiography (C and D).

N964 Opa$^-$ P$^+$, which binds to epithelial cells without significant invasion, did not induce PC consumption or DAG release (Figures 1B and 1C). The PC-PLC activity was mediated by a mammalian PC-PLC, since experiments measuring the activity of PC-PLC by consumption of [^{14}C]dipalmitoyl-phosphatidylcholine did not show any activity in N242 Opa$_{27.5}$, whereas PC-PLC-positive Listeria monocytogenes degraded radioactive PC. A possible involvement of phospholipase D (PLD), which is also inhibited by D609 (Kiss and Tomono, 1995), in the invasion process of Ngo was ruled out, as no release of phosphatidic acid, no transphosphatidylation by PLD, and no effect of butan-1-ol on the synthesis of DAG after cellular infection with N242 Opa$_{27.5}$ was observed (data not shown).

Invasive N. gonorrhoeae Trigger Release of Ceramide via Activation of PC-PLC and ASM

Several studies have implicated a regulation of ASM by PC-PLC-released DAG (Schütze et al., 1992; Wiegmann et al., 1994). We therefore tested whether invasive Ngo activate ASM, which catalyzes the release of ceramide from sphingomyelin. Infection of Chang cells with N242 Opa$_{27.5}$ resulted in a rapid, ~35% reduction of labeled, cellular SM (Figure 2A), correlating with an ~3-fold increase of ceramide release, which was inhibited by D609 (Figures 2A–2C). This implies an important function of the PC-PLC in Ngo-induced SM turnover. The integrin-binding E. coli strain H1422 Inv$^+$ or the adherent, noninvasive gonococcal strain N964 Opa$^-$ P$^+$ did not induce any change of SM or ceramide (Figures 2A–2C), demonstrating the specificity of the observed reaction for gonococcal invasion.

Ceramide could be released from SM by a neutral sphingomyelinase and/or ASM (Hannun, 1996). We observed a 2.5-fold stimulation of ASM activity in immunoprecipitates as well as in cell lysates after infection with the invasive strains N242 Opa$_{27.5}$ and N483 Opa$_{30}$, whereas H1422 Inv$^+$ and N964 Opa$^-$ P$^+$ did not alter cellular ASM activity (Figures 3A and 3B). Consistent with the effects of D609 on SM consumption and ceramide release, this drug also blocked the activation of ASM upon bacterial internalization (Figures 3A and 3B).

In contrast to the activation of ASM, no stimulation of the neutral sphingomyelinase activity was observed after infection with N242 Opa$_{27.5}$ or H1422 Inv$^+$ (101% ± 3% or 98% ± 4%, respectively, compared with the activity in uninfected cells). However, the neutral sphingomyelinase was activated in Chang cells ~1.6-fold (165% ±

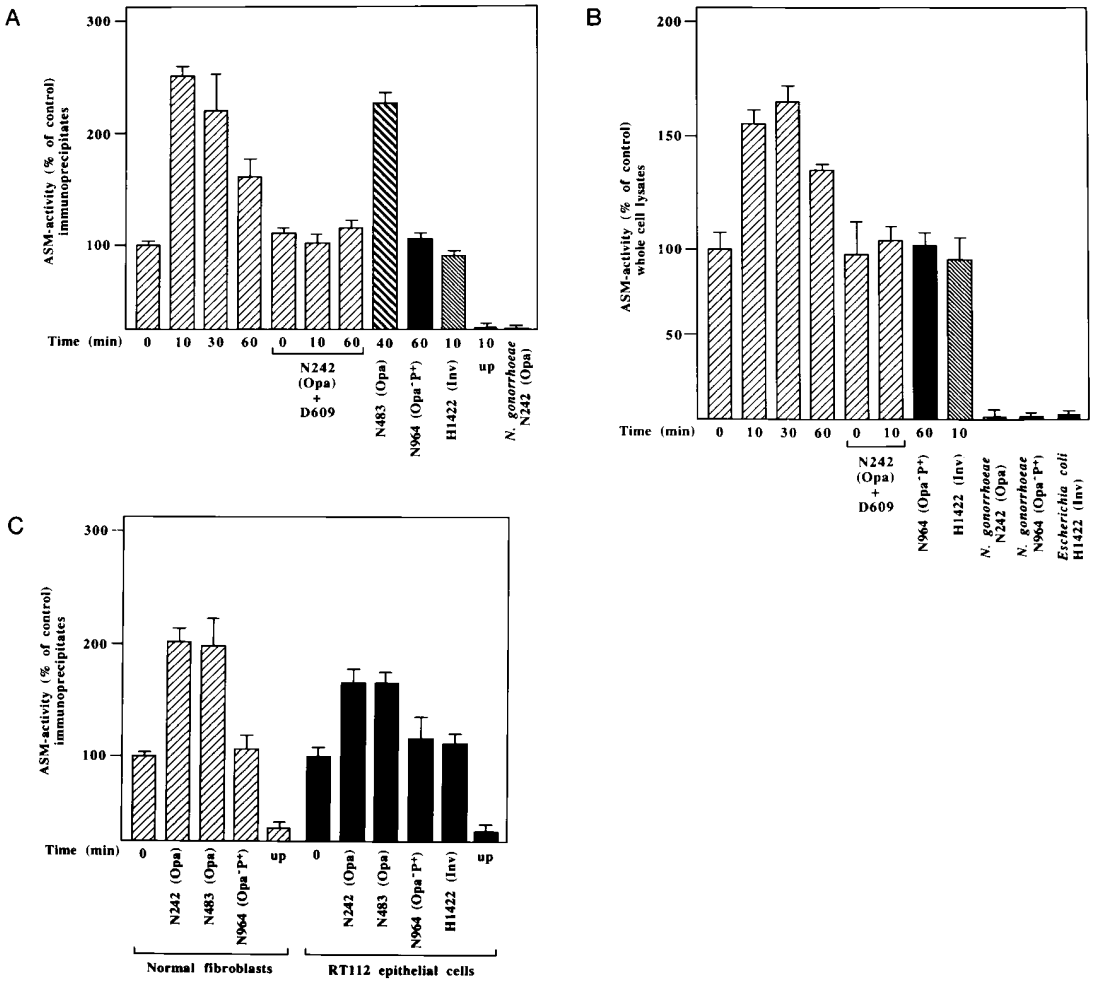

Figure 3. Internalization of Gonococci Stimulates the Acidic Sphingomyelinase

Invasion of Chang and RT112 cells or fibroblasts by the HSPG-specific strain N242 $Opa_{27.5}$ or N483 Opa_{30} activates the ASM, whereas N964 $Opa^- P^+$ or H1422 Inv^+ did not trigger ASM. ASM activity was determined by degradation of [^{14}C]SM after immunoprecipitation (A and C) or in cell extracts (B) and was inhibited by preincubation with D609 (A–C). Total counts obtained with immunoprecipitates from noninfected samples were ~10,000 cpm/10^6 cells; in the lysates, ~11,000 cpm/10^6 cells. No intrinsic bacterial ASM activity could be detected in immunoprecipitates or in bacterial lysates. Unspecific immunoprecipitates (up) were performed with a normal goat anti-human Ig-antiserum followed by addition of protein A/G. Total counts were 30–50 cpm/10^6 cells. Shown is the mean ± SD of three independent experiments.

12%) upon activation of the cells with tumor necrosis factor α (TNFα), showing that the neutral sphingomyelinase can principally be activated in these cells.

Since Ngo N964 $Opa^- P^+$ required more time (~60 min) to adhere efficiently to Chang cells as compared to N242 $Opa_{27.5}$ (~10 min), we determined the critical role of the ASM pathway for gonococcal invasion also by the use of normal human fibroblasts and the bladder epithelial cell line RT112, which show for both gonococcal strains a rapid onset and similar quantity of adhesion. Both fibroblasts and RT112 cells responded with a stimulation of ASM to infection with invasive N242 $Opa_{27.5}$ or N483 Opa_{30} but not with the noninvasive strain N964 (Figure 3C), showing the close link between ASM activation and internalization of Ngo.

ASM Activation Is Required for N. gonorrhoeae Entry into Nonphagocytic Cells

To define the significance of ASM activation and ceramide release for Opa-mediated Ngo uptake, we tested whether ASM-deficient NPDA fibroblasts or imipramine-treated Chang cells are susceptible to infection. Imipramine has been shown to induce proteolysis of the active 72 kDa ASM form via an unknown mechanism, whereas the inactive 75 kDa form remains unaffected (Hurwitz et al., 1994a), which was confirmed in Chang cells by Western blotting of immunoprecipitated ASM (Figure 4A, inset). This blot also demonstrates that D609 did not change ASM protein levels in Chang cells. Jurkat earlier shown to express ASM served as positive control (Gulbins et al., 1995). The decrease in protein level upon imipramine treatment correlated with a 85% reduction of ASM activity in immunoprecipitates (data not shown). Pharmacological suppression of ASM by imipramine prevented internalization of N242 $Opa_{27.5}$ by Chang cells in a dose-dependent manner (Figure 4A), correlating with a prevention of SM degradation or ceramide release. As shown in light microscopy and gentamicin survival studies, imipramine did not change adherence to host cells and had no obvious effect on the viability

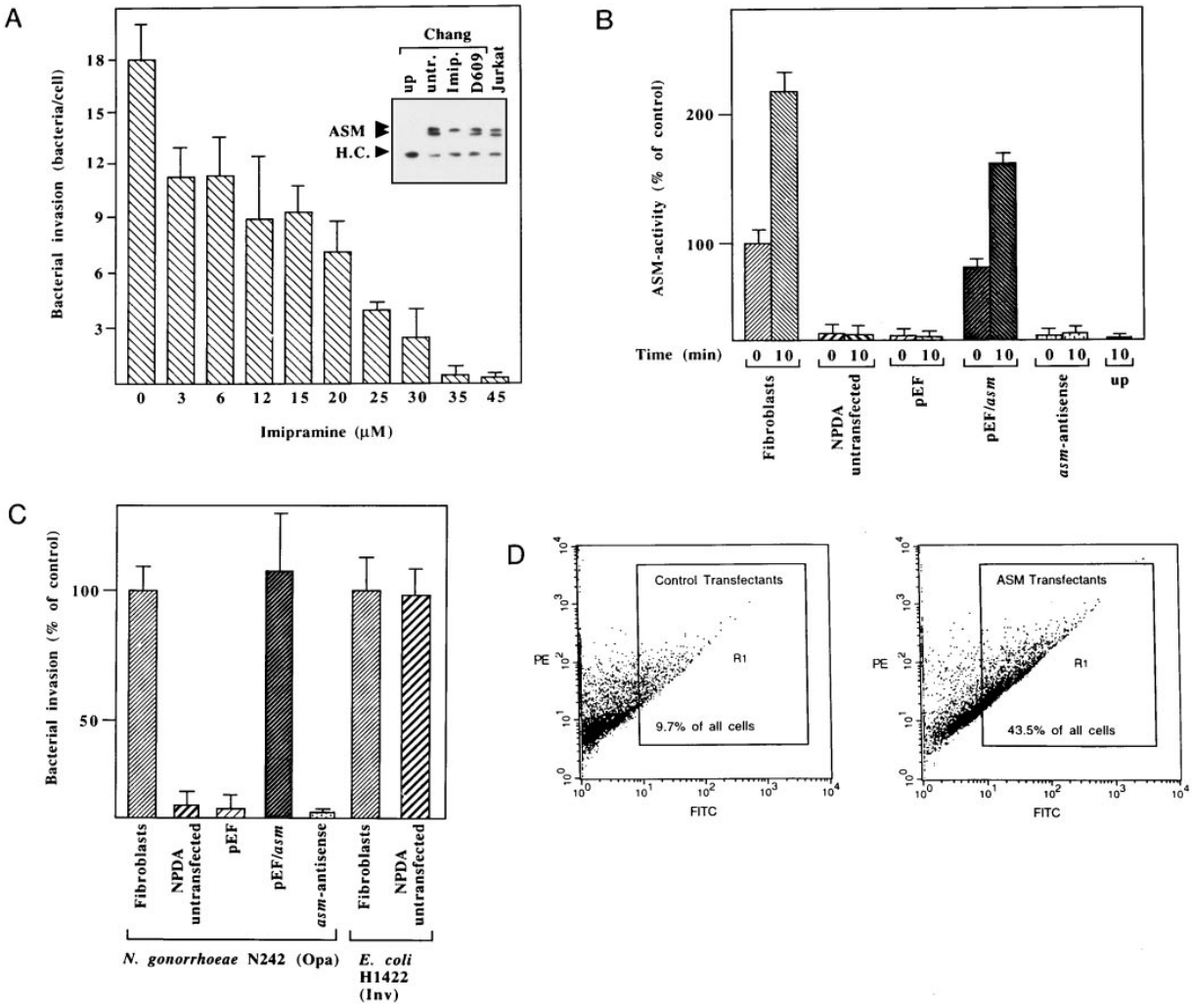

Figure 4. Acidic Sphingomyelinase Is Required for the Uptake of N. gonorrhoeae into Nonphagocytic Cells

(A) Degradation of ASM by imipramine induced a dose-dependent inhibition of N242 $Opa_{27.5}$ uptake of Ngo into target cells without affecting the adherence as determined by crystal violet assays. Degradation of the active 72 kDa ASM isoform by imipramine was confirmed by immunoprecipitation and Western blotting of ASM (inset) as well as by direct measurement of ASM activity (data not shown). The strong band at ~55 kDa is the heavy chain of the antibody used for immunprecipitation (H. C.).

(B) ASM-deficient NPDA fibroblasts were transiently transfected with an expression vector for ASM (pEF-*asm* or pJK-*asm*), an antisense construct of ASM (*asm*-antisense), or with vector control (pEF, pJK). Transfection restored the activity level of the ASM to ~75% of normal fibroblasts in response to an infection with N242 $Opa_{27.5}$ for 10 min. ASM activity was determined as above. Up, unspecific immunoprecipitates.

(C and D) Invasion of Ngo is prevented by genetic deficiency of ASM in untransfected, vector control, or *asm* antisense transfected NPDA fibroblasts. Transfection of ASM reconstitutes uptake of N242 $Opa_{27.5}$ into the cells. In contrast, the invasin-triggered uptake of the E. coli strain H1422 Inv^+ is not affected by genetic deficiency of ASM (C). The invasion of N242 $Opa_{27.5}$ and H1422 Inv^+ was determined by gentamicin assays (C) or FACS analysis (D) 36 hr after transfection. R1 in the FACS blots indicates the gate to determine FITC-positive, i.e., infected, cells. The according percentage is shown in the blot. The results show that Ngo internalization depends on functional ASM expression.

of either bacteria or epithelial cells. The inhibitory effect of imipramine on gonococcal uptake is supported by additional findings from our group showing that 30 μM of SR33557, another ASM inhibitor (Higuchi et al., 1996), also inhibited N242 $Opa_{27.5}$ invasion and ASM activity in Chang cells by 65%–80% (T. Rudel, personal communication).

To assess further the significance of ASM activation for Ngo uptake, ASM-deficient NPDA fibroblasts were used. The NPDA fibroblasts were reconstituted for ASM by transfection with a mammalian expression vector encoding ASM (pEF-*asm*), vector alone, or with a construct expressing antisense *asm*. Normal human fibroblasts were used as positive control. The activity level of ASM in the reconstituted NPDA cells reached ~75% of the level in the same amount of normal fibroblasts, while ASM activity was undetectable in nontransfected, vector control, or antisense *asm*–transfected NPDA cells (Figure 4B).

Internalization of N242 $Opa_{27.5}$ was strictly dependent on ASM expression, since bacterial invasion was observed only upon infection of normal fibroblasts and the ASM reconstituted NPDA fibroblasts (Figure 4C). In contrast, almost no uptake could be detected in untransfected, vector control, or antisense *asm*–transfected NPDA cells (Figure 4C). To exclude the possibility that an increased sphingomyelin content in cell membranes of NPDA fibroblasts results in a general defect

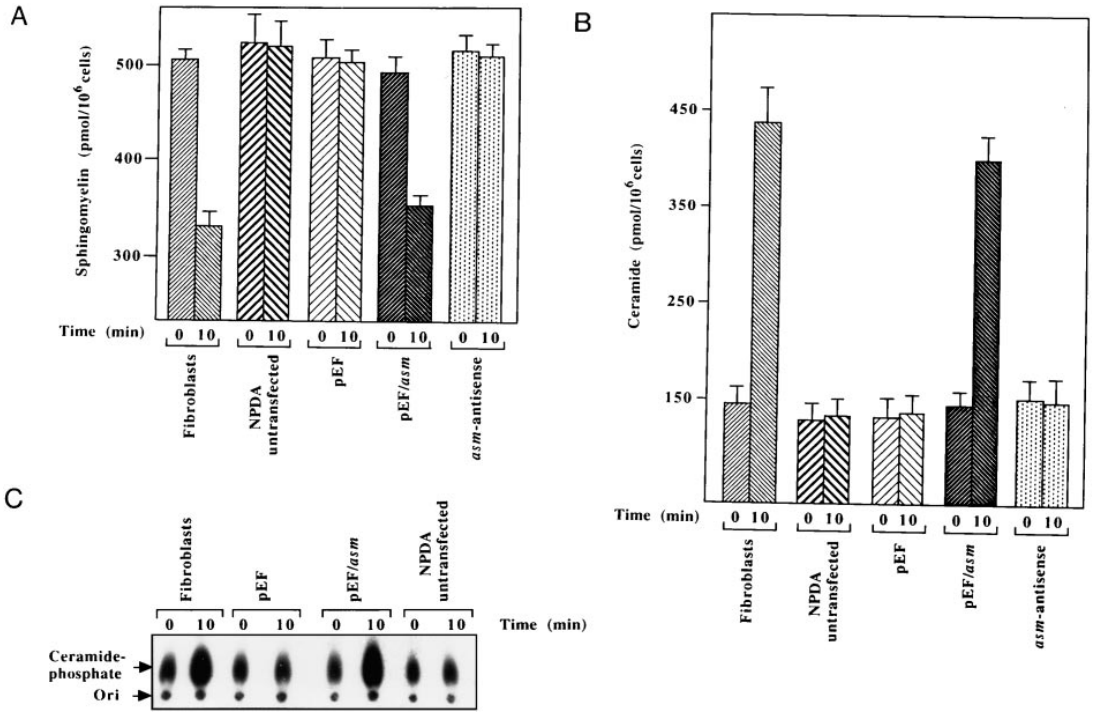

Figure 5. Sphingomyelin Consumption and Ceramide Release in Response to N. gonorrhoeae Invasion Depend on the Function of ASM

Genetic deficiency of ASM prevents degradation of SM (A) and ceramide release (B and C) upon infection with N242 $Opa_{27.5}$. Transfection of ASM restores these signaling events (A–C). SM consumption or ceramide release was determined in normal fibroblasts or in genetically deficient NPDA fibroblasts left either untransfected or transfected with expression vectors for ASM (pEF-*asm*), the control vector pEF, or an antisense construct of *asm* as described. (B) shows the LSC of radioactive ceramide-1-phosphate and (C) an autoradiography of a representative experiment.

of bacterial or receptor internalization, we measured the invasion of the integrin receptor-binding E. coli strain H1422 Inv^+ (Figure 4C). This bacterium showed a normal uptake in NPDA fibroblasts, demonstrating that these cells are prinicipally able to internalize bacteria and/or receptors. The absolute requirement of ASM expression for Ngo invasion was confirmed with a second NPDA fibroblast line obtained from a different patient.

In addition, NPDA fibroblasts were transiently transfected with a plasmid (20 μg) encoding ASM and simultaneously a *myc*-tagged immunoglobulin heavy chain (pJK-*asm*), infected with N242 $Opa_{27.5}$, and stained with anti-Myc 9E10 antibody followed by a phycoerythrine (PE)-conjugated antibody. Extracellular Ngo were blocked with a large excess of a polyclonal Ngo antiserum (AK213) followed by incubation with an unconjugated antiserum. Cells were then permeabilized using 0.2% Triton X-100, incubated with AK213, and intracellular bacteria were stained with fluorescein isothiocyanate (FITC)-coupled antiserum. The fluorescence-activated cell sorter (FACS) results (Figure 4D) showed that an infection (FITC signal) was almost exclusively observed in pJK-*asm*-transfected NPDA cells (PE signal). The results further demonstrated that transfection of ASM-deficient NPDA fibroblasts results in a clear increase of the number of infected cells from ~10% in the vector control pJK-transfected samples to ~40% infected cells upon transfection with pJK-*asm*. The transfection efficiency of ~40% was confirmed by transfection of a CD20 expression construct (pRc/CMV-*cd20*). CD20 is not expressed on untransfected Chang cells or NPDA fibroblasts. Finally, transfection of normal Chang cells with pJK-*asm* resulted in an ~2-fold increase of the bacterial invasion rate, suggesting that ASM is rate-limiting with regard to gonococcal uptake (data not shown).

The prevention of gonococcal uptake by genetic ASM deficiency in NPDA fibroblasts correlated with a failure to respond with SM degradation (Figure 5A) or ceramide release (Figures 5B and 5C) upon infection by invasive N242 $Opa_{27.5}$. In contrast, normal fibroblasts and ASM-reconstituted NPDA cells responded to N242 $Opa_{27.5}$ infection with SM consumption (Figure 5A) and ceramide formation (Figures 5B and 5C). In summary, these data point to a crucial function of ASM in Opa/HSPG-mediated entry of Ngo into nonphagocytic cells.

Discussion

A common theme of bacterial cell invasion mechanisms appears to be the activation of host signal transduction pathways. Our results establish a genetic link between ASM activation and internalization of Ngo into several nonphagocytic human cell types and demonstrate a new function of the ASM-dependent signaling pathway (Figure 6). The crucial function of ASM for gonococcal uptake is indicated by (1) the consumption of SM, (2) the

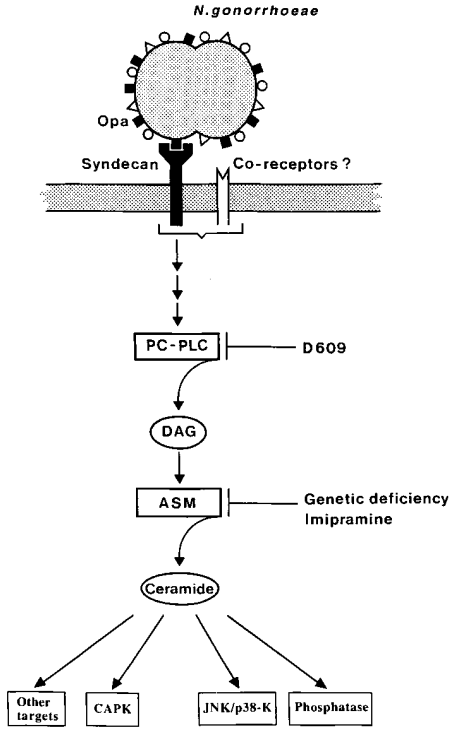

Figure 6. Model of the Signaling Cascade Initiated by and Required for the Internalization of N. gonorrhoeae into Nonphagocytic Cells

Binding of invasive Ngo to the putative receptor molecule directly or indirectly induces an activation of the PC-PLC, which catalyzes the release of DAG from PC. DAG seems to activate ASM, resulting in consumption of SM and release of ceramide. Genetic deficiency of ASM or pharmacological inhibition of PC-PLC or ASM prevents internalization of invasive Ngo, showing the crucial function of this signaling pathway for gonococcal uptake. Ceramide finally may stimulate a variety of target molecules.

release of ceramide, (3) the stimulation of ASM in immunoprecipitates as well as in whole-cell lysates, and (4) the inhibition of N242 $Opa_{27.5}$ invasion by genetic ASM deficiency or imipramine-mediated blockade of ASM. In contrast, Ngo did not activate the neutral sphingomyelinase, which has been shown to respond to the adhesion of uropathogenic E. coli with ceramide release (Hedlund et al., 1996). Thus, our data point to a specific involvement of the ASM for bacterial internalization. To confirm further the release of ceramide upon Ngo infection, we showed that Ngo infection triggered an ASM-dependent activation of JNK, a known downstream target of ceramide (Westwick et al., 1995; Verheij et al., 1996).

The activation of PC-PLC or ASM does not seem to be related to the adhesion of Ngo, since the piliated Ngo strain N964 $Opa^- P^+$ did not induce PC-PLC or ASM stimulation. N242 $Opa_{27.5}$ and N964 $Opa^- P^+$ adhered with the same efficiency to RT112 endocervical epithelial cells or fibroblasts, while the latter strain N964 $Opa^- P^+$ showed essentially no invasion. D609 reduced the binding of Ngo to epithelial cells by 25%–30%, which is insufficient to explain the 95% blockage of gonococcal uptake by the epithelial cells upon D609 treatment, supporting the notion that PC-PLC is required for internalization rather than adhesion of the bacteria.

Our data do not exclude other mechanisms of gonococcal uptake. In particular, vitronectin strongly facilitates HSPG-mediated gonococcal invasion of certain human epithelial cell lines including HeLa but not Chang (Duensing and van Putten, 1997; Gómez-Duarte et al., 1997). Since vitronectin binds integrins, this mechanism probably triggers an alternative, ASM-independent gonococcal entry. As shown here, H1422 Inv^+ is efficiently internalized by NPDA fibroblasts. Therefore, uptake via integrins does not seem to involve ASM. Moreover, our preliminary data indicate that invasion of Ngo in HeLa cervical or Hec1B endometrial epithelial cells does not result in an activation of ASM. Thus, different internalization pathways in different cell types seem to exist for Opa-expressing HSPG-specific gonococci, probably dependent on nature and quantitative distribution of receptor molecules. Further, Ngo-expressing CD66-specific Opa proteins may enter epithelial cells by utilizing additional pathways (Chen and Gotschlich, 1996; Virji et al., 1996; Bos et al., 1997; Gray-Owen et al., 1997).

A similar signaling cascade involving PC-PLC, ASM, and JNK has been described after cellular stimulation via the IL-1β (Mathias et al., 1993), TNFα (Schütze et al., 1992; Wiegmann et al., 1994), CD95 (Cifone et al., 1994; Gulbins et al., 1995; Tepper et al., 1995), or CD28 receptor (Boucher et al., 1995). These observations raise the question of how receptors inducing such diverse biological functions activate the same signaling pathway. Thus, ASM activation by these receptors may have a dual function: first, ASM might be important for the internalization of some of these receptors, and Ngo may utilize this pathway to gain access to the intracellular compartment. The early stimulation of ASM suggests a direct involvement in bacterial uptake and a role in phagosome formation. The notion of a function of ASM for internalization of receptors belonging to the TNF/NGF receptor family is supported by a recent report showing internalization of herpes simplex virus 1 by binding a member of this receptor class (Montgomery et al., 1996). Second, in addition to the involvement in internalization of pathogens or receptor molecules, ASM and ceramide have been implicated in the regulation of cellular apoptosis (Cifone et al., 1994; Gulbins et al., 1995; Tepper et al., 1995; Hannun, 1996; Verheij et al., 1996). Therefore, the release of ceramide in epithelial cells upon gonococcal infection may finally induce programmed cell death, permitting gonococci to cross the epithelial border and to colonize submucosal tissues.

The finding that Ngo invade several nonphagocytic cell types via activation of the ASM pathway establishes a genetic link between expression and activation of ASM and internalization of invasive pathogens. Several other bacteria including Yersinia enterocolitica and pseudotuberculosis, Shigella flexneri, Salmonella typhimurium, Listeria monocytogenes, or enteropathogenic E. coli have been shown to employ protein tyrosine kinase-linked pathways (Galán et al., 1992; Rosenshine et al., 1992a, 1992b; Bliska et al., 1993; Dehio et al., 1995; Mengaud et al., 1996; Watarai et al., 1996), activation of phospholipase A_2 (Galán et al., 1992; Pace et al., 1993), phosphatidylinositide-3-kinase (Ireton et al., 1996), or small G proteins, in particular CDC42Hs, Rho, and Rac (Adam et al., 1996; Chen et al., 1996), respectively, for

uptake. Whether the activation of ASM and the release of ceramide upon bacterial invasion is a completely independent pathway or interacts with the described signaling events remains to be investigated.

Our results with genetically ASM-deficient cells as well as pharmacological inhibitors of PC-PLC and ASM suggest a signaling cascade from a putative receptor molecule binding Ngo to the activation of PC-PLC and the release of DAG. DAG stimulates ASM via unknown mechanisms resulting in the release of ceramide (Figure 6). The data suggest a crucial role of the described pathway in the infection with Ngo. Since HSPG receptors are essential for the interaction of gonococci with epithelial cells (Chen et al., 1995; van Putten and Paul, 1995), this interaction may trigger the ASM pathway. Consequently, other HSPG-specific pathogens, including viruses (Shieh et al., 1992; Compton et al., 1993) or some other bacteria (Liang et al., 1992; Zhang and Stephens, 1992; Issacs, 1994), might also induce the ASM pathway. It is, however, possible that other adhesive factors expressed in gonococci in addition to the HSPG-specific Opa proteins, such as the glycolipid adhesins or LPS (Paruchuri et al., 1990; van Putten, 1993), may actually be responsible for stimulating the described pathway.

Experimental Procedures

Bacterial Strains

N242 $Opa_{27.5}$ is a nonpiliated variant of Ngo strain VP1 (P^-, serotype P1.A, LPS type L13) expressing the invasion-associated $Opa_{27.5}$ protein (Makino et al., 1991; van Putten, 1993) resembling the recombinant Opa_{68} protein (Kupsch et al., 1993). N483 Opa_{30} (MS11–B2.1) is a nonpiliated spontaneous mutant of strain MS11 (P^-, *pilE1*, and *pilE2*, serotype P.IB, LPS type A) expressing the invasion-associated Opa_{30} protein (Makino et al., 1991) resembling the recombinant Opa_{60} (Kupsch et al., 1993). N964 is a piliated derivative of Ngo MS11 (Opa^- P^+, $PilE_{F3}$, *recA::cat*, serotype P.IB, LPS type A) (Rudel et al., 1992; van Putten, 1993). Gonococci were grown for ~18 hr on GC agar base (Life Technologies, Eggenstein, Germany) with 1% vitamin mix at 37°C in a humidified atmosphere containing 5% CO_2. H1422, an E. coli DH1 derivative that contains the *inv* gene of Y. pseudotuberculosis cloned in pACYC184 was generated according to the method of Isberg et al. (1987) and grown on LB supplemented with chloramphenicol (30 μg/ml) at 37°C.

Cell Culture

Chang human conjunctiva epithelial cells (ATCC CCL20.2) were cultured in RPMI 1640 (Life Technologies) supplemented with 5% fetal calf serum (FCS). The human urinary bladder carcinoma cell line RT112, kindly provided by Dr. W. Franke, DKFZ, Heidelberg, Germany, was maintained in Waymouth's MB752, 2 mM L-glutamine, and 10% FCS. ASM-deficient fibroblasts were obtained from two different patients with NPDA exhibiting <1% residual ASM activity. Niemann-Pick patients suffer from an inborn defect of functional ASM expression (Brady et al., 1966). Normal human fibroblasts were obtained from PromoCell GmbH, Heidelberg, Germany, or cultured directly from skin biopsies of healthy donors (100% ASM activity). ASM-deficient and control fibroblasts were maintained either in MEM, 10% FCS, 2 mM L-glutamine, 100 μM nonessential amino acids, 100 U/ml penicillin, and 100 μg/ml streptomycin or in fibroblast growth medium (serum-free), supplemented with growth factors and antibiotics (PromoCell). For infection experiments, cells were seeded 48 hr prior to the assay onto appropriate tissue culture plates.

Infection Experiments

Plate-grown bacteria were suspended in RPMI 1640 culture medium supplemented with 50 mM HEPES (pH 7.4), shook for 2 hr at 135 rpm (37°C), pelleted, and resuspended in fresh RPMI 1640. Target cells were washed with RPMI 1640 and maintained in the same medium during the infection experiment. Infection was initiated by inoculating subconfluent cell layers at a bacteria (N242 $Opa_{27.5}$ or H1422 Inv^+) host cell ratio of 10:1 (for crystal violet assay), 75:1 (for lipid isolation or immunoprecipitation studies of epithelial cells), or 800:1 (for all fibroblast experiments), respectively. Ngo N964 Opa^- P^+ were inoculated at a ratio of 750:1 for Chang and of 25:1 for RT112 cells. The bacteria host cell ratio for N483 Opa_{30} was 100:1 for epithelial cells. The different ratios were chosen to obtain a similar number of adhering bacteria. To achieve synchronous infection conditions and to enhance the bacteria host cell interaction, bacteria were centrifuged for 2 min onto target cells (35 × g). The end of the centrifugation was defined as start (zero) point in all infection assays. Infection was terminated by washing the cells three times with phosphate-buffered saline (PBS) followed by lipid extraction for SM, PC, DAG, or ceramide measurements, fixation in PBS containing 1% paraformaldehyde for crystal violet assays, lysis in PBS containing 1% saponin for gentamicin assays, or lysis in ice-cold sonication buffer (see below) for determining ASM activity. D609 (Calbiochem, Bad Soden, Germany) or imipramine (Sigma, Deisenhofen, Germany) was added 15 or 30 min prior to infection; control cells were treated with the same amount of solvent. To exclude possible effects of the inhibitors on the bacteria, the ability of the pathogens N242 $Opa_{27.5}$ and H1422 Inv^+ to invade epithelial cells was measured after withdrawal of the inhibitor. Further, bacteria were incubated with the inhibitors in the absence of human cells, and the ability to infect untreated epithelial cells after inhibitor removal was determined by crystal violet and gentamicin assays.

Internalization and Survival Assays

Bacterial invasion was assessed either by crystal violet staining or in gentamicin survival assays. For crystal violet staining, 2 hr infected cells (2×10^5/well) were fixed with 1% paraformaldehyde in PBS for at least 15 min at 20°C and stained overnight with 0.07% crystal violet in H_2O. Intracellular versus adherent bacteria were microscopically counted from at least 50 cells as described (van Putten et al., 1990).

The number of live intracellular bacteria was determined by survival of gentamicin treatment (Makino et al., 1991). Infected cells were washed three times with PBS, cultured in RPMI 1640 supplemented with 100 μg/ml gentamicin for 2 hr, washed and lysed in 1% saponin for 10 min at 37°C, plated, and colonies were counted after 48 hr. Values of both assays are presented as mean ± SD of at least three independent experiments.

Phosphatidylcholine and Sphingomyelin Consumption

Epithelial cells (~8×10^6/plate) or fibroblasts (~4×10^5/plate) were metabolically labeled by incubation with 1 μCi/ml [methyl-^{3}H]choline chloride (60–90 Ci/mmol; NEN-DuPont, Germany) for 48 hr in complete RPMI 1640 or MEM medium, washed, and infected for the indicated time. Infection was terminated by washing the cells in PBS and scraping them into a lysis buffer consisting of 0.05% SDS, 1% Triton X-100, 10 mM EDTA, 10 mM sodium-pyrophosphate, 10 mM sodium fluoride, 25 mM HEPES, 125 mM NaCl, and 10 μg/ml each aprotinin and leupeptin (A/L). Samples were normalized for protein and radioactivity, and lipids were extracted by addition of 120 μl of 0.22 M HCl, 2.7 ml of $CHCl_3$:CH_3OH (2:1), 0.9 ml of $CHCl_3$, and 0.9 ml of 1 M KCl. The organic phase was dried, resuspended in $CHCl_3$:CH_3OH (1:1), and lipids were separated on G60 silica gel TLC plates (Machery-Nagel, Germany) with $CHCl_3$:CH_3OH:H_2O:acetic acid (50:30:8:4). PC and SM were identified in I_2 vapor by comigration with standards, scraped from the plate, and radioactivity was determined by LSC.

Activity of Acidic and Neutral Sphingomyelinase

The activity of ASM upon cellular infection was measured by immunoprecipitation as well as in whole-cell lysates. Epithelial cells (~8×10^6/plate) or fibroblasts (~4×10^5/plate) were infected, washed, and lysed in ice-cold 50 mM Tris (pH 7.4), 1 mM each bacitracin, benzamidine, Na_3VO_4, 10 μg/ml each A/L, 0.1 mg/ml soybean trypsin inhibitor, and 0.2% Triton X-100 (sonication buffer). Samples were immediately sonicated three times for 10 s each. Insoluble cell debris

was pelleted by 5 min centrifugation at 600 × g, an equal amount of 50 mM Tris (pH 7.4), 3% NP40, 1% Triton X-100, 1 mM Na_3VO_4, and 100 μg/ml each A/L (ASM lysis buffer) was added to the samples, and ASM was immunoprecipitated using a previously characterized goat-anti-ASM serum (Hurwitz et al., 1994b) followed by protein A/G-coupled agarose (Santa Cruz, Inc., Santa Cruz, CA). Samples were washed three times each in ASM lysis buffer and in 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, 1 mM Na_3VO_4, and 10 μg/ml each A/L followed by incubation with [^{14}C]SM (0.5 μCi/sample, 54.5 mCi/mmol; NEN-DuPont) in 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA, and 0.05% NP40 (assay buffer) for 30 min at 37°C. The substrate [^{14}C]SM was solubilized after drying by 10 min bath sonication in assay buffer. Samples were then extracted with $CHCl_3$:CH_3OH (2:1) and H_2O, the upper phase was collected, and radioactivity reflecting the degradation of [^{14}C]SM was determined by LSC.

ASM activity in whole-cell lysates was determined by scraping the cells into 0.1% Triton X-100, 50 mM sodium acetate (pH 5.0), 0.1 mM Na_3VO_4, and 10 μg/ml each A/L. Extracts were sonicated, centrifuged at 600 × g for 5 min, the supernatants were added to the same volume of 0.5 μCi/sample [^{14}C]SM in assay buffer, and samples were processed as above.

For determination of neutral sphingomyelinase activity, cells were scraped into 20 mM HEPES (pH 7.4), 5 mM dithiothreitol, 2 mM EDTA, 10 mM $MgCl_2$, 0.1 mM Na_2MoO_4, 10 mM β-glycerophosphate, 7.5 mM ATP, 1 μM PMSF, 0.2% Triton X-100, and 10 μM leupeptin. Extracts were processed as above with 0.5 μCi/sample [^{14}C]SM as substrate.

Determination of Diacylglycerol and Ceramide

For determination of ceramide, infected epithelial cells (~8 × 10^6/plate) or fibroblasts (~4 × 10^5/plate) were washed and lysed by scraping the cells into 25 mM HEPES, 0.05% SDS, 1% Triton X-100, 10 mM EDTA, 10 mM each sodium-pyrophosphate and sodium fluoride, 10 μg/ml each A/L, and 125 mM NaCl. Samples were normalized for protein, and the lipids were extracted with $CHCl_3$:CH_3OH:HCl (100:100:1). The organic phase was dried under vacuum, DAG was digested by alkaline hydrolysis in 0.1 M methanolic KCl, the samples were reextracted as above, dried, and solubilized by 10 min sonication in 7.5% (w/v) n-octyl-β-glucopyranoside, 5 mM cardiolipin, 1 mM diethylenetriamine-pentaacetic acid. After sonication, 40 μg/ml purified E. coli DAG kinase (Amersham, Germany) in 70 μl of reaction buffer (100 mM imidazole-HCl [pH 6.6], 100 mM NaCl, 25 mM $MgCl_2$, and 2 mM EGTA) supplemented with 10 μCi/sample [γ-^{32}P]ATP and 1 mM ATP was added, and the samples were incubated at 20°C for 30 min. Kinase reaction was stopped by addition of 1 ml of $CHCl_3$:CH_3OH:HCl (100:100:1), 170 μl of salt solution (10 mM HEPES [pH 7.2], 135 mM NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 5.6 mM glucose), and 30 μl of 100 mM EDTA. The lower organic phase was dried, resuspended in $CHCl_3$:CH_3OH (1:1), and resolved by TLC using $CHCl_3$:CH_3OH:acetic acid (65:15:5) as solvent. Spots were visualized by autoradiography, scraped from the plate, and analyzed by LSC.

The release of DAG was determined by the same protocol with omission of the alkaline hydrolysis in 0.1 M methanolic KCl. The ceramide or DAG content of the samples was determined by comparison with a standard concentration curve of DAG or ceramide.

Direct release of DAG or ceramide was measured by labeling Chang cells with 1 μCi/ml [^{14}C]lyso-palmitoylphosphatidylcholine (57 mCi/mmol, NEN-DuPont) for 12 hr or with 2 μCi/ml [^{3}H]serine (27 mCi/mmol, NEN-DuPont) for 24 hr, respectively. After infection, DAG was extracted with 1 ml of $CHCl_3$:CH_3OH (1:2) followed by the addition of 0.33 ml each of 1 M NaCl and $CHCl_3$. Ceramide was extracted with $CHCl_3$:CH_3OH:H_2O:pyridine (60:160:6:1). The lower phases were dried, resuspended in $CHCl_3$:CH_3OH (95:5), and lipids were separated on silica G60 HPTLC plates (Merck) using benzene:$CHCl_3$:CH_3OH (80:15:5) for DAG or $CHCl_3$:CH_3OH:$CaCl_2$ (60:35:8) for ceramide. For ceramide measurements, phospholipids were degraded with methanolic NaOH at 37°C for 2 hr prior to TLC. DAG or ceramide was visualized by autoradiography.

Phosphatidic acid release was determined by infection of [^{14}C] lyso-palmitoylphosphatidylcholine-labeled Chang cells. Samples were extracted with 1 ml of $CHCl_3$:CH_3OH (1:2), 0.33 ml each of 1 M NaCl and $CHCl_3$, dried, separated by TLC with $CHCl_3$:CH_3OH:propan-1-ol:0.25% KCl:ethylacetate (25:13:25:9:25), and analyzed by autoradiography and LSC. To determine the transphosphatidylation activity, samples were incubated with 20 mM butan-1-ol 5 min prior and during the infection.

Transfection of ASM-Deficient NPDA Fibroblasts

ASM-deficient NPDA fibroblasts were transiently transfected with an expression vector directing the expression of ASM under the control of an elongation factor promoter (pEF). Control experiments were performed with the pEF vector and with an antisense construct of ASM. For transfection, cells (~10^5/well) were incubated with 30 μg of pEF-*asm*, vector control pEF, or antisense *asm* and 10 μl/ml lipofectamine in Optimem medium (Life Technologies) for 8 hr. Transfection was terminated by adding the same volume of cell culture medium supplemented with 20% FCS. ASM or control transfected cells were used for infection experiments as described above 36 hr after transfection. Bacterial invasion was assessed by gentamicin survival assays.

In order to detect transiently transfected cells by FACS analysis, cells were transfected with a vector encoding both the *asm* under control of the EF promoter and, under control of a CMV promoter, a construct consisting of a single-chain antibody fused with a Myc tag (designated as pJK-*asm*). This construct also encodes a single-chain immunoglobulin sequence consisting of a transmembranous and an extracellular domain.

FACS Analysis

Cellular transfection was detected by incubation of the cells with an anti-Myc tag 9E10 antibody (Sigma) followed by staining with a PE-conjugated antiserum (Dianova). Intracellular bacteria were visualized after permeabilization using an anti-Ngo antibody (AK213) and a FITC-conjugated antiserum (Dianova). FACS analysis was performed on a Becton Dickinson Calibur.

Acknowledgments

We would like to thank Drs. K. Coggeshall, S. Gutkind, and M. Kroenke for valuable reagents and Drs. C. Dehio, S. Gray-Owen, C. Hauck, M. Naumann, T. Rudel, and W. van den Akker for critical comments on the manuscript and for kindly carrying out supplementary experiments with the ASM inhibitor SR33557. We greatly acknowledge the excellent technical help of A. Behyl, J. Kun, and A. Riehle. The study was supported by DFG grants Gu335/2-2, La315/6-1, and AICR grant 96-18 to E. G., SFB400 (A15) to K. S., and the Fonds der Chemischen Industrie to T. F. M.

Received May 6, 1997; revised October 8, 1997.

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