CD66-mediated phagocytosis of Opa52 *Neisseria gonorrhoeae* requires a Src-like tyrosine kinase- and Rac1-dependent signalling pathway

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The interaction of *Neisseria gonorrhoeae* with human phagocytes is a hallmark of gonococcal infections. Recently, CD66 molecules have been characterized as receptors for Opa52-expressing gonococci on human neutrophils. Here we show that Opa52-expressing gonococci or *Escherichia coli* or F(ab) fragments directed against CD66, respectively, activate a signalling cascade from CD66 via Src-like protein tyrosine kinases, Rac1 and PAK to Jun-N-terminal kinase. The induced signal is distinct from Fcγ-receptor-mediated signalling and is specific for Opa52, since pilated Opa gonococci, commensal *Neisseria cinea* or *E. coli* do not stimulate this signalling pathway. Inhibition of Src-like kinases or Rac1 prevents the uptake of Opa52 bacteria, demonstrating the crucial role of this signalling cascade for the opsonin-independent, Opa52/CD66-mediated phagocytosis of pathogenic *Neisseria*. Keywords: gonococci/phagocytes/Rac1/signal transduction/Src-like kinases

Introduction

Gonorrhoea, caused by the human-specific, Gram-negative pathogen *Neisseria gonorrhoeae*, is often characterized by a cervical or urethral purulent exudate consisting mainly of human phagocytic cells (Handsfield, 1990). Phagocytosis of the bacteria seems to be a crucial event in the symptomatic disease process, resulting in killing of the gonococci by the phagocytes. However, some of the phagocytes also contain obviously viable gonococci, which may be protected against the bactericidal mechanisms of the infected cell and may use the phagocytes as an intracellular niche and as vehicles to reach the next host (Shafer and Rest, 1989; Meyer et al., 1994). Elucidation of the molecular mechanisms underlying phagocytosis of *N. gonorrhoeae*, therefore, could help to unravel the strategy employed by this extreme host-adapted organism to survive in the presence of numerous human immune effector cells.

The uptake of particles by phagocytes involves opsonin-independent as well as opsonin-dependent pathways. One of the best characterized opsonin-dependent pathways is the Fcγ-receptor-mediated internalisation of antibody-coated particles or bacteria (Sheterline et al., 1984; Salmon et al., 1991; Zheleznyak and Brown, 1992; Davis et al., 1995). In this case, phagocytosis of bacteria is mediated by specific antibodies linking the bacterial surface to phagocyte Fcγ-receptors. With regard to this uptake process, the tyrosine kinase Syk plays a central role (Greenberg et al., 1994; Matsuda et al., 1996) and seems to be sufficient for mediating particle internalisation via Fcγ-receptors (Greenberg et al., 1996).

In contrast, several gonococcal surface proteins have been shown to be involved in the efficient opsonin-independent uptake of *N. gonorrhoeae* by professional and non-professional phagocytes, since internalization takes place in the absence of specific antibodies or complement (Swanson et al., 1975; Bessen and Gotschlich, 1986; Virji and Heckels, 1986; Fischer and Rest, 1988; Rest and Shafer, 1989). These bacterial proteins belong to the opacity (Opa) protein family and are responsible for the opaque colony phenotype of goncocci. Eleven different Opa proteins have been detected in *N. gonorrhoeae* MS11, which are subject to genetic variation (Stern et al., 1986; Bhat et al., 1992; Robertson and Meyer, 1992; Kupsch et al., 1993). They are integral proteins of the outer membrane and mediate the intimate contact between the bacteria and their host cells (Weel et al., 1991). The variation of Opa proteins is crucial for the change of target cell tropisms in *N. gonorrhoeae* (Kupsch et al., 1993). In this regard, the Opa50 protein promotes bacterial invasion into epithelial cells, whereas Opa52 and Opa60 mediate an opsonin-independent uptake by professional phagocytes (Fischer and Rest, 1988; Elkins and Rest, 1990; Makino et al., 1991; Kupsch et al., 1993). The tropism differences conferred by the Opa proteins have recently been defined on the molecular level, in that Opa50 was shown to recognise heparan sulfate containing surface proteoglycan receptors on human epithelial cells (Chen et al., 1995; van Putten and Paul, 1995), whereas Opa52 and Opa60 specifically interact with CD66 antigens (Chen and Gotschlich, 1996; Virji et al., 1996; Gray-Owen et al., 1997).

CD66 antigens belong to a family of plasma membrane-linked glycoproteins often referred to as the carcinoembryonic antigen (CEA) family, with several members expressed on cells of the myeloid lineage including biliary glycoprotein (BGP), non-specific cross reacting antigen (NCA), CEA gene family member 1 (CGM1) and CGM6 (Thompson et al., 1991; Gray-Owen et al., 1997). Though the exact function of CD66 family members is currently unknown, they have been shown to mediate homo- and heterotypic cell–cell interaction (Benchimol et al., 1989; Oikawa et al., 1992). Recently, it has been demonstrated that CD66 associates with tyrosine kinases of the Src family in neutrophils, implying an involvement of these kinases in CD66-mediated cell signalling (Brunner et al., 1995; Skubitz et al., 1995).
In the present study, we provide evidence for a CD66-triggered signalling cascade in phagocytes during opsonin-independent phagocytosis of \textit{N. gonorrhoeae} strain MS11 (N309 Opa\textsubscript{52}). Uptake of gonococci by phagocytes induces the activation of the Src-family tyrosine kinases Hck and Fgr and the phosphorylation of several cellular proteins. The stimulation of Hck and Fgr results in an activation of the small G-protein Rac1, its substrate PAK and, finally, Jun-N-terminal kinase (JNK). Inhibition of this signalling cascade prevents the internalization of N309 Opa\textsubscript{52} by phagocytes showing the biological significance of the observed activation events for the infection. These data support the notion that Opa-dependent phagocytosis is an actively induced process that may facilitate the intracellular accommodation and survival of \textit{N. gonorrhoeae} in professional phagocytes as an essential step during the course of an infection.

**Results**

\textit{Neisseria gonorrhoeae} producing Opa\textsubscript{52} stimulates tyrosine phosphorylation in phagocytic human cells

The present study was aimed to identify molecular mechanisms involved in the opsonin-independent phagocytosis of pathogenic, Opa\textsubscript{52}-expressing \textit{N. gonorrhoeae}. Tyrosine phosphorylation is one of the key events in signal transduction pathways mediating bacterial internalisation (Bliska et al., 1993). Therefore, we investigated the tyrosine phosphorylation pattern induced by the non-piliated (P\textsuperscript{−}) Opa\textsubscript{52}-expressing strain N309. Since gonococcal Opa\textsuperscript{−} P\textsuperscript{−} variants do not interact with human phagocytes in the absence of opsonins (Fischer and Rest, 1988; Kupsch et al., 1993; Hauck et al., 1997; Knepper et al., 1997), we used the N280 Opa\textsuperscript{−} P\textsuperscript{+} variant of \textit{N. gonorrhoeae} MS11 for comparison with N309 Opa\textsubscript{52}. This variant can interact with phagocytic cells via pili (Hauck et al., 1997; Knepper et al., 1997), allowing us to discriminate between pilus or Opa protein-induced signals. In addition, a commensal \textit{Neisseria cinerea} strain N340 was used as a control in order to investigate the specificity of the induction pattern for \textit{N. gonorrhoeae}.

Incubation of in vitro-differentiated JOSK-M cells with N309 Opa\textsubscript{52} in the absence of opsonins resulted in increased tyrosine phosphorylation of several proteins already after 10 min with a maximum after 30 min (Figure 1). In particular, proteins with an apparent molecular weight of 110–120 kDa, 95 kDa, 65 kDa, 55 kDa, 43 kDa, 32 kDa and 24 kDa showed a pronounced increase in tyrosine phosphorylation in response to Opa\textsubscript{52}-expressing gonococci, whereas proteins with an apparent molecular weight of 53 kDa, 50 kDa and 27 kDa showed no change in intensity of tyrosine phosphorylation. JOSK-M cells not stimulated with bacteria showed the same tyrosine phosphorylation pattern and intensity as cells at the 0 min time point (not shown). In contrast, cells treated with either the broad spectrum protein kinase inhibitor staurosporine or the Src-like tyrosine kinase inhibitor herbimycin A, respectively, lacked some of the tyrosine phosphorylated proteins found in unstimulated cells. The increase in protein tyrosine phosphorylation induced by N309 Opa\textsubscript{52} was inhibited by preincubation of JOSK-M cells with the kinase inhibitors (Figure 1). To demonstrate the significance of Opa\textsubscript{52} in the induction of tyrosine phosphorylation, the \textit{N. cinerea} strain N340 or the N280 Opa\textsuperscript{−} P\textsuperscript{+} variant were incubated with the phagocytic cells. Neither of these triggered a significant increase in cell tyrosine phosphorylation (Figure 1) and phosphorylation levels of the proteins were comparable to zero time of Opa\textsubscript{52}-stimulated cells.

**Opsonin-independent internalisation of N309 Opa\textsubscript{52} is mediated by CD66 and depends on tyrosine kinases**

To investigate the function of CD66 and the significance of cellular tyrosine phosphorylation for the uptake of Opa\textsubscript{52} \textit{N. gonorrhoeae} by JOSK-M cells, cells were preincubated with anti-CD66 antibodies or with kinase inhibitors, and infected for 1 h with N309 Opa\textsubscript{52}, N280 Opa\textsuperscript{−} P\textsuperscript{+}, \textit{N. cinerea} N340 or \textit{Escherichia coli} H1887. Only N309 Opa\textsubscript{52} was efficiently internalised by the phagocytic cells in the absence of opsonins during the 1 h infection period (Figure 2A). In contrast, only a small population of the phagocytes carried internalised N280 Opa\textsuperscript{−} P\textsuperscript{+}, \textit{N. cinerea} N340 or \textit{E. coli} H1887 at this time point. This is in accordance with our previous studies (Hauck et al., 1997; Knepper et al., 1997), where we observed a significantly enhanced phagocytosis of N309 Opa\textsubscript{52} by \textit{in vitro}-differentiated JOSK-M cells or primary neutrophils compared to N280 Opa\textsuperscript{−} P\textsuperscript{+} and \textit{N. cinerea} N340 after 4 h (JOSK-M cells) or 1 h (neutrophils) of infection.

Treatment with polyclonal rabbit anti-CD66 antibodies completely blocked internalisation of N309 Opa\textsubscript{52}, demonstrating the pivotal role of the Opa\textsubscript{52}/CD66 interaction for the uptake process. Inhibition of cellular kinases by staurosporine correlated with a complete blockade of the Opa\textsubscript{52}-dependent phagocytosis of \textit{N. gonorrhoeae}. Likewise, the Src-like tyrosine kinase inhibitor herbimycin A...
Neisseria phagocytosis via CD66, Src-like kinases and Rac1

Fig. 2. Opsonin-independent phagocytosis of N.gonorrhoeae N309 Opa52 via CD66 depends on a herbimycin-A-sensitive mechanism. (A) N309 Opa52 was efficiently phagocytosed in the absence of opsonins, whereas N280 Opa–P, N.cinerea N340 or E.coli H1887 were not taken up within the 1 h infection period. Anti-CD66 antibodies (αCD66; 50 μg/ml), the kinase inhibitor staurosporine (+Stau; 1 μM) or the Src kinase inhibitor herbimycin A (+Herb; 5 μM) almost completely blocked internalisation of N309 Opa52. Infection was analysed by double-immunofluorescence staining of bacteria and human lysosome-associated membrane protein 2 (h-lamp-2), and subsequent determination of the ratio of infected cells. Bars represent mean values ± standard deviations of at least three experiments. (B) After immunofluorescence staining, intracellular Opa52 N.gonorrhoeae (arrowhead) could be readily differentiated from extracellular bacteria (arrow) by their co-localisation with the phagosomal/lysosomal marker protein h-lamp-2, resulting in a yellow halo in the pseudocolor overlay of the laser scanning confocal microscope images. Although the kinase inhibitors herbimycin A (+Herb) and staurosporine (+Stau) both blocked the uptake of N309 Opa52, bacteria still adhered to the cells (arrows). Goncocci appear green and h-lamp-2 appears red in the pseudocolored images. Scale bars represent 10 μm.

Phagocytosed N309 Opa52 bacteria could be detected within h-lamp-2 positive phagosomes, whereas cells treated with staurosporine or herbimycin A did not contain intracellular bacteria (Figure 2B). Importantly, the adherence of N309 Opa52 to JOSK-M cells was not affected by either inhibitor.

Activation of Src-like protein tyrosine kinases in response to N309 Opa52

Since herbimycin A specifically inhibits Src-like protein tyrosine kinases, we measured the activation level of Hck or Fgr, the Src-family kinases known to be expressed predominantly in human phagocytes (Ziegler et al., 1987; Ley et al., 1989; Xu et al., 1996), upon infection of JOSK-M cells by immunoprecipitation and immunocomplex kinase assays. Consistent with the specific tyrosine phosphorylation induced by N309 Opa52 and the inhibition of the uptake of these bacteria by herbimycin A, only N309 Opa52 stimulated Hck and Fgr, whereas neither N280 Opa–P, N.cinerea N340, nor E.coli H1887 activated these kinases (Figure 3A and B). Staurosporine or herbimycin A pretreatment of the phagocytes abolished Hck and Fgr activation in response to N309 Opa52. Control immunoprecipitations from cells infected for 30 min with N309 Opa52 using irrelevant affinity purified rabbit antibodies demonstrated the specificity of the immunoprecipitation
Fig. 3. The Src-like kinases Hck and Fgr are activated during phagocytosis of *N. gonorrhoeae* MS11 Opas52. (A) and (B) Immunoprecipitation and immunocomplex kinase assays of Hck (A; upper panels) and Fgr (B; upper panel) revealed a strong induction of kinase activity during phagocytosis of N309 Opas52. Preincubation with herbimycin A (+Herb; 5 μM) or staurosporine (+Stau; 1 μM) inhibited the activation of the kinases. Neither N280 Opas52, *N. cinerea* N340, nor *E. coli* H1887 stimulated kinase activity. Unspecific control immunoprecipitates (*) were performed from cells infected with N309 Opas52 for 30 min using an irrelevant polyclonal rabbit antibody. Aliquots of the immunoprecipitates were blotted with anti-Hck or anti-Fgr, respectively, to demonstrate similar protein levels in all lanes (A and B; lower panels). Experiments were repeated at least three times with similar results. (C) Phagocytosis of N309 Opas52 induced a translocation of Hck to the phagosome. Cells were infected with N309 Opas52 for 60 min, fixed and stained with antibodies to *N. gonorrhoeae* MS11 (α-*N. gonorrhoeae*; green) and Hck (α-Hck; red). In contrast to extracellular bacteria (arrow), intracellular bacteria (arrowhead) co-localised with intense accumulations of the kinase as demonstrated by the yellow halo in the pseudocolor overlay of the laser scanning confocal microscope images. Scale bar represents 5 μm. (D) Activation of Hck by N309 Opas52 was already observed at a m.o.i. of 5. A lower bacteria:cell ratio did not lead to significant activation of Hck (upper panel). Western blotting of the immunoprecipitates demonstrated similar amounts of kinase in the samples (lower panel).
Involvement of the small GTP-binding protein Rac1 in the uptake of N309 Opa52

Phagocytosis is dependent on cytoskeletal rearrangements which are known to be regulated by small GTP-binding proteins of the Rho-subfamily of Ras-related proteins (Hall, 1994; Zigmond, 1996). These small G-proteins seem to be controlled, at least under certain conditions, by Src-like tyrosine kinases (Crespo et al., 1997). We therefore investigated whether the GTPases Rac1 and Cdc42Hs were activated by N309 Opa52. Infection of JOSK-M cells with N309 Opa52 induced a strong stimulation of Rac1 as determined by the increase in the GTP/GDP-ratio bound to Rac1 (Figure 4A). This stimulation could be blocked by pretreatment of the cells with staurosporine. N280 OpaP+ or N. cinnerea N340 had no effect on Rac1-bound GTP level (Figure 4A).

In contrast to Rac1, Cdc42Hs was not activated by N309 Opa52 (not shown). The kinetics of Rac1 stimulation resembled the kinetics of protein tyrosine phosphorylation (Figure 1A) as well as Hck and Fgr activation (Figure 3A and B). To analyse the mechanism of Rac1 activation in response to N309 Opa52, JOSK-M cells were pretreated with herbimycin A. This Src-specific inhibitor completely blocked Rac1 stimulation, indicating that the GTPase acts downstream of the protein tyrosine kinases Fgr and/or Hck (Figure 4B).

To investigate the function of Rac1 in phagocytosis of N309 Opa52, we inhibited Rac1 expression by incubation of JOSK-M cells for 48 h with phosphorothioate oligonucleotides complementary to the 5′ end of Rac1 or Cdc42Hs mRNA coding sequence, or with control oligonucleotides containing the same nucleotides in a randomised order. The Rac1 antisense oligonucleotide reduced Rac1 protein expression levels by ~90%, whereas the control oligonucleotide did not alter Rac1 protein levels (Figure 4C inset). Cells treated with Rac1 antisense oligonucleotides showed a significantly reduced internalization of N309 Opa52 compared with cells which received the control oligonucleotide (Figure 4C). No effect was seen with the antisense-Cdc42Hs or randomised control oligonucleotides. Even though the antisense oligonucleotides reduced Rac1 expression by 90%, the remaining cellular protein seems still to be sufficient for a considerable uptake of the bacteria. This might be due to an efficient recruitment of Rac1 to the site of bacterial internalisation and, in spite of the overall reduction, relatively high local concentrations of this protein.

The stress-activated protein kinase pathway is induced by N309 Opa52 via Src-like kinases

The serine/threonine kinase PAK has recently been described as being regulated by small GTPases of the Rho-subfamily (Manser et al., 1994). Therefore, we measured the activity of PAK by immunoprecipitation from Neisseria-infected JOSK-M cells and immunocomplex kinase assays. PAK-mediated phosphorylation of myelin basic protein (MBP) was enhanced by N309 Opa52, whereas N280 OpaP+ or N. cinnerea N340 did not induce PAK activity (Figure 4D). Preincubation of the phagocytes with herbimycin A (Figure 4D) or staurosporine (not shown) abolished activation of PAK. The kinetics of PAK stimulation paralleled the pattern observed for tyrosine phosphorylation as well as Hck, Fgr and Rac1 activation.

Several groups have established that the activation of Rac proteins is linked to the regulation of stress-activated protein kinases or Jun-N-terminal kinases (JNK) and p38 kinase (p38-K) (Cosso et al., 1995; Minden et al., 1995). JNK was strongly stimulated upon infection of JOSK-M cells with N309 Opa52 (Figure 4E). Activation of JNK was restricted to infection by Opa52-producing bacteria and was inhibited by pretreatment of the cells with herbimycin A (Figure 4E) or staurosporine (not shown).

Opas52-induced stimulation of Src-like tyrosine kinases and JNK is mediated via CD66

It has been shown that Opa52 selectively binds to CD66 family members (Gray-Owen et al., 1997). To further demonstrate the specificity of the observed signalling cascade for Opa52 proteins, we incubated phagocytic cells with recombinant E.coli DH5α producing Opa52 (H1907; Figure 5A). Activation of Hck or JNK was observed only after incubation of JOSK-M cells with H1907 Opa52, whereas Opa-negative E.coli (H1887) failed to induce these activation events (Figure 5A and B), indicating a crucial role for the Opa52/CD66 interaction in the observed signalling cascade.

To further demonstrate that the described pathway is distinct from Fcγ receptor-mediated cell activation, we determined the activity of Syk, a tyrosine kinase known to be stimulated during Fcγ receptor-mediated uptake (Greenberg et al., 1994). N309 Opa52 failed to stimulate Syk, whereas gonococci opsonised with normal human serum induced an 8-fold activation of Syk (Figure 5C), supporting the notion that the uptake of N. gonorrhoeae via Opa52/CD66 interaction can be clearly differentiated from Fcγ receptor-mediated internalisation.

To study the role of CD66 in the activation of Hck and JNK, we investigated whether cellular activation via CD66 results in a similar signalling cascade as observed after infection with N309 Opa52. To this end, JOSK-M cells were stimulated with Fab fragments of a polyclonal rabbit anti-CD66 antibody. Fab fragments were used to prevent an involvement of Fcγ receptor-mediated cell activation. The results show a strong activation of Hck and JNK upon cellular triggering via CD66 (Figure 5D and E). The activation of the kinases was independent of clustering of CD66 molecules, since activation was observed even without addition of mouse anti-rabbit F(ab)2-fragments. Addition of irrelevant F(ab)2-fragments did not stimulate the kinases (Figure 5D and E).

Hck and JNK are induced in primary human neutrophils upon contact with N309 Opa52

To demonstrate the physiological significance of the described pathway observed in the phagocytic JOSK-M cell line, we tested Fgr and JNK activation in primary
Fig. 4. Activation of the small G-protein Rac1, PAK and JNK and the essential role of Rac1 for the internalisation of *N. gonorrhoeae* MS11 Opa52. (A) Cellular infection with N309 Opa52 induced activation of the small G-protein Rac1, as visualised by the increase in GTP eluted from Rac1. In contrast, N280 Opa P- or *N. cinerea* N340 did not stimulate Rac1 as determined by the GTP/GDP ratio bound to Rac1. Preincubation with the broad specific kinase inhibitor staurosporine (+Stau; 1 μM) prevented Rac1 activation. A control immunoprecipitation (*) was performed from cells infected for 30 min with N309 Opa52, using an irrelevant polyclonal rabbit antibody. (B) The specific Src-like tyrosine kinase inhibitor herbimycin A abolished Rac1 stimulation during N309 Opa52 infection. Pretreated (+Herb; 5 μM) or untreated cells were infected with N309 Opa52, lysed and Rac1 was immunoprecipitated. Rac1-bound guanine nucleotides were eluted and separated by TLC followed by autoradiography. The increase of Rac1-bound GTP upon infection reflects the stimulation of this small G-protein. (C) Inhibition of Rac1 expression by incubation with antisense oligonucleotides reduced infection of JOSK-M cells with N309 Opa52. Cells were incubated with 60 μM Rac1 or Cdc42Hs antisense (α) or control oligonucleotides (α*) for 48 h, infected for 2 h with N309 Opa52, and phagocytosis was determined by double-immunofluorescence staining of bacteria and h-lamp-2. Bars represent mean values ± standard deviations of three independent experiments. The inset shows the expression level of Rac1 in antisense- (α) or control-oligonucleotide- (α*) treated cells and demonstrates the efficiency of the antisense oligonucleotide treatment. (D) and (E) The p21-activated protein kinase PAK (D) and JNK (E) were strongly stimulated by cellular infection with N309 Opa52, but not upon infection with N280 Opa P- or *N. cinerea* N340. Pretreatment of the cells with herbimycin A (+Herb; 5 μM) completely blocked PAK or JNK activation (D and E, upper panel). PAK or JNK were immunoprecipitated and kinase activity was determined by phosphorylation of the substrate MBP or GST-c-Jun, respectively, followed by separation on SDS–PAGE, blotting and autoradiography. The blots were stripped and reprobed to show similar amounts of protein present in all lanes (D and E, lower panel). Non-specific immunoprecipitations (*) were performed using irrelevant polyclonal rabbit antibodies. Experiments were repeated twice (PAK) or three times (JNK).

Discussion

Though phagocytosis is a key step of innate immunity, the cellular signalling involved in the uptake by professional phagocytes has so far only been studied in detail using antibody-opsonized particles (Zheleznyak and Brown, 1992; Greenberg et al., 1994; Davis et al., 1995; Matsuda et al., 1996). Since some bacteria are also internalized without prior opsonisation by complement or specific antibodies, this type of phagocytosis could be important during initial steps of the infection.

Recently, CD66 molecules have been demonstrated to function as surface receptors for gonococci expressing distinct opacity (Opa) outer membrane proteins (Chen and Gotschlich, 1996; Virji et al., 1996; Gray-Owen et al.,
Neisseria phagocytosis via CD66, Src-like kinases and Rac1

Fig. 5. Activation of Hck and JNK upon N. gonorrhoeae internalisation is mediated by Opas2 binding to CD66, independent of Syk activation, and mimicked by cellular triggering with anti-CD66 F(ab)-fragments. (A) Infec-
tion of JOSK-M cells with E.coli DH5α Opas2 (H1907) stimulated Hck to a similar extent as N309 Opas2. E.coli DH5α containing the empty vector (H1887) did not activate Hck. The lower panel shows a Hck Western blot demonstrating equal amounts of immunoprecipitated kinase. (B) Stimulation of JNK in response to H1907 Opas2 was comparable to stimulation by N309 Opas2 (upper panel). E.coli H1887 did not stimulate JNK activity. Western blotting with a JNK-antibody (lower panel) demonstrated equal amounts of immunoprecipitated kinase. (C) Stimulation of cells with opsonised, but not with non-opsonised, N309 Opas2 resulted in activation of Syk. N309 Opas2 was opsonised by incubation with normal human serum (NHS) or left untreated. JOSK-M cells were stimulated with opsonised or un-opsonised gonococci or with NHS and lysed after the indicated times. Syk was immunoprecipitated from post-centrifugation supernatants and activity of Syk was determined by autophosphorylation upon addition of [γ-32P]ATP to the immunoprecipitates followed by SDS–PAGE and autoradiography. Western blotting demonstrated equal amounts of immunoprecipitated Syk kinase in all samples. All experiments were repeated twice. (D) and (E) The signalling pathway stimulated by N. gonorrhoeae N309 Opas2 is mimicked by cellular triggering with anti-CD66 F(ab)-fragments. Cells were incubated with anti-CD66 F(ab)-fragments (α-CD66) on ice for 15 min. Mouse anti-rabbit-F(ab)2-fragments (α-rabbit) were added as indicated and samples were incubated at 37°C for various periods of time. The activities of Hck (D) or JNK (E) were determined by immunocomplex kinase assays as described above (upper panels). Control immunoprecipitations (*) with irrelevant antibodies were performed on samples stimulated with both antibody preparations. Samples were separated by SDS–PAGE and analysed by autoradiography. Lower panels show Western blots of the immunoprecipitates and demonstrate equal amounts of kinases in the samples. Representative blots from three independent experiments are shown.

1997). Several members of the CD66 family are expressed on human phagocytes and have been shown to be associated with tyrosine kinases (Brummer et al., 1995; Skubitz et al., 1995). We previously showed efficient uptake of N. gonorrhoeae MS11 Opas2 (N309) as well as E.coli DH5α Opas2 (H1907) by CD66 transfected HeLa cells (Gray-Owen et al., 1997). In addition, phagocytosis is inhibited by anti-CD66 antibodies, indicating that Opas2-mediated binding to CD66 is the trigger for the opsonin-independent uptake of these bacteria. Therefore, by using MS11 Opas2 or DH5α Opas2, we were able to analyse CD66-mediated cellular activation events in human phagocytes. The results point to a new signalling pathway from Src-like tyrosine kinases via Rac1 and PAK to JNK initiated by CD66 receptor molecules (Figure 7). This pathway is crucial for the internalization of N. gonorrhoeae N309 Opas2, since inhibition of Src-like tyrosine kinases by the specific inhibitor herbimycin A or suppression of Rac1 by antisense oligonucleotides (reducing Rac1 protein expression level by ~90%) prevent or reduce N309 Opas2 uptake. The activation of the observed pathway was mimicked by anti-CD66 antibodies; thus a physiological CD66 ligand may elicit a similar signalling cascade, thereby mediating biological functions of CD66 surface receptors. Since the expression of CD66 molecules has been demonstrated to negatively regulate growth of colon carcinoma cells (Kunath et al., 1995) or prostate carcinoma cells (Hsieh et al., 1995) in mice, the identification of a CD66-initiated signalling pathway may also stimulate future studies on the role of this signal in suppression of tumour growth.

The failure of a N. gonorrhoeae MS11 Opa+ P+ variant (N280), commensal N. cinerea N340 or E.coli H1887 to induce the observed responses demonstrates the specificity of the signalling pathway for the Opa52/CD66 interaction. In addition, it argues against the participation of LPS or LOS in the observed induction of protein tyrosine phosphorylation (Weinstein et al., 1991) or in stimulation of the stress-activated protein kinase JNK during the in vitro infection. Some elements of the observed pathway, in particular the stimulation of Hck, seem to be common for both uptake mechanisms, opsonin-independent via...
CD66 or opsonin-dependent via Fcγ receptors (Wang et al., 1994). However, the specific effect of a certain molecule, in this case Hck, depends to a great extent on the context and the recruitment of other signalling molecules by the involved receptor. This is underscored by the lack of Syk activation during Opa52-dependent internalisation of distinct kinase subsets coupling to different pathways or the specific combination of certain signalling molecules.

Representative blots from two independent experiments are shown.

Fig. 6. Activation of Src-like kinases and JNK by N.gonorrhoeae N309 Opa52 in primary human polymorphonuclear granulocytes. PMN were obtained from healthy donors, infected with N309 Opa52 or N280 Opa*. Phosphorylation of Hck (A) or JNK (B) was determined as described above (upper panels). Unspecific control immunoprecipitations (*) with irrelevant antibodies contained no detectable kinase activity. The lower panels show similar amounts of kinases in all samples with specific immunoprecipitations.

Recently, Beauchemin and co-workers (1997) showed that the cytoplasmic tail of mouse CD66a (BGP) contains an Immunoreceptor Tyrosine-based Inhibition Motif (ITIM) mediating a tyrosine-phosphorylation-dependent association of mouse BGP with the tyrosine phosphatase SHP-1. The phosphatase SHP-1 has been implicated in the down-regulation of cytotoxic functions in natural killer cells (Binstadt et al., 1996) and it may be responsible for inhibition of bactericidal mechanisms after the uptake of Opa52-expressing N.gonorrhoeae. Stimulation of this pathway may result in reorganisation of the cytoskeleton as a prerequisite for bacterial internalisation and/or in the regulation of gene expression or induction of apoptosis.
suggest that different pathogens employ similar but distinct pathways for internalisation.

GTP-loaded Rac1 interacts with several downstream effectors (Manser et al., 1994; Martin et al., 1996; van Aelst et al., 1996). Though PAK binds to activated Rac1, recent investigations demonstrated that the activity of the PAK kinase is not correlated with the cytoskeletal rearrangements induced by Rac1 (Joneson et al., 1996; Lamarche et al., 1996; Sells et al., 1997) and rather is linked to the activation of JNK (Bagrodia et al., 1995), supporting the proposed signalling pathway (Figure 7).

The selective uptake of Opa52 gonococci via CD66 molecules and the generation of a distinct signalling cascade from Hck/Fgr, Rac1 and PAK to JNK points towards a specific function of this pathway. Interestingly, the major portion of gonococci recovered from human volunteers experimentally infected with OpaN. gonorrhoeae MS11 expressed an Opa52-homologue (Swanson et al., 1988). Thus, expression of the Opa52 outer membrane protein seems to confer a selective survival advantage. It could be speculated that the internalization of gonococci via a receptor which is responsible for homo- and heterotypic cell–cell interaction (Benchimol et al., 1989; Oikawa et al., 1992) and which associates with inhibitory signalling molecules like SHP-1 (Beauchemin et al., 1997) targets the pathogens to a different intracellular compartment than bacteria phagocytosed via CD66 may allow for prolonged intracellular survival of the bacteria. In addition to directing the bacteria to a specific intracellular compartment, the observed signalling pathway might lead to the stimulation of members of the AP-1 transcription complex via activated JNK, resulting in an altered gene expression in phagocytes infected with Opa52 gonococci. Finally, JNK has been implicated in playing an important role in stress-induced apoptosis, e.g. upon cellular irradiation, heat shock or treatment with UV-light (Chen,Y.R. et al., 1996; Rosette and Karin, 1996; Verheij et al., 1996; Ichijo et al., 1997). Thus, pathogenic Opa52 gonococci may promote apoptotic processes of their host cell upon internalisation via the activation of a signalling cascade leading to JNK stimulation.

Given the induction of a specific signal transduction pathway during CD66-mediated uptake and the potential role the activated molecules play in the host cell, it is intriguing to speculate that surface expression of Opa52 by gonococci is a means to enter professional human phagocytes on a route that circumvents bactericidal mechanisms of these specialised host cells.

Materials and methods

Cells and culture

The human myelomonocytic cell line JOSK-M (Ohta et al., 1986) was obtained from the German Collection for Microorganisms (DSM ACC330) and grown as suspension in RPMI1640 (Life Science Technologies) supplemented with 5% fetal calf serum (Boehringer Mannheim), 2 mM t-glutamine at 37°C, 5% CO2. Cells were subcultured every 3–4 days. Prior to infection with gonococci, JOSK-M cells were differentiated in vitro by the addition of retinoic acid (100 nM final concentration) and bufalin (10 nM final concentration) to the culture medium, and incubation of the cells for 5–7 days.

Western blotting and FACS analysis with the monoclonal antibodies CLB/gran10 recognising CD66a, CD66c, CD66d, CD66e (Hiss Diagnostics), 80H3 detecting CD66b (Immunotech), 26/3/13 recognising CD66e and 9A6 detecting CD66c (generous gifts of Dr Fritz Grunert, Freiburg) revealed that only differentiated JOSK-M cells expressed CD66b, CD66d and CD66e.

Human neutrophils were isolated from peripheral blood of healthy volunteers essentially as described by Brandt et al. (1991) to minimise pre-activation of the cells.

Viability of JOSK-M cells and neutrophils was determined prior to infection using trypan blue staining and was >95% in all cases. As indicated, cells were preincubated with staurosporine (1 μM) for 15 min, herbinycin A (5 μM) for 12 h or polyclonal anti-CD66 antibodies (50 μg/ml; azide deLETED; Dako, Hamburg, Germany) for 5 min prior to infection.

Bacteria

The gonococcal variants MS11-B1 (N273; PiElEg, P5) and MS11-F3 (N238; PiElEg P+) (Haas et al., 1987) are the progenitors of the strains used in the present work and are derived from N.gonorrhoeae MS11 (Meyer et al., 1984). N280, a piliated strain exhibiting the transparent phenotype (Opa– P+), as well as the non-piliated strain N309 expressing a phagocytose-Specific Opa protein (Opa52 P) have been previously described (Kupsch et al., 1993). All gonococci were grown on GC-agar (Life Science Technologies) supplemented with vitamins and corresponding antibiotics at 37°C in 5% CO2 and subcultured daily. Commensal N.cinerea (N340) was obtained from U.Berger, Heidelberg, grown on GC-agar and subcultured daily. Recombinant E.coli DH5α producing Opa52 (H1907) and DH5β harbouring the empty expression vector pTec99A (H1887) have also been described by Kupsch et al. (1993). The E.coli strains were maintained on LB-plates supplemented with 100 μg/ml ampicillin.

Infection of the cells

Plate-grown bacteria were suspended in RPMI1640 and washed by centrifugation at 4000 r.p.m. for 5 min in a microcentrifuge. After resuspension in RPMI1640 supplemented with 550 nm (gonococci) or 600 nm (E.coli) in a DR2000 spectrophotometer (Hach, Coveland, CO), and bacteria were added to JOSK-M cells or primary neutrophils in RPMI1640 supplemented with 5% heat inactivated FCS at a ratio of 50 bacteria/cell at 37°C to start the infection. In some cases, gonococci were opsonized with 1% pooled normal human serum FCS at a ratio of 50 bacteria/cell at 37°C to start the infection. In some cases, gonococci were opsonized with 1% pooled normal human serum (NHS) for 15 min at 37°C prior to infection. After the indicated time, cells were pelleted by centrifugation at 250 g for 2 min at 4°C (for immunoprecipitation), or washed twice with PBS for 5 min at 120 g in a microcentrifuge and then centrifuged on glass coverslips for 5 min at 50 (for immunofluorescence staining).

Cellular tyrosine phosphorylation and immunoblotting

Cellular infection was terminated by lysis in 25 mM HEPES (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl, 10 mM each of NaF, Na3VO4 and sodium pyrophosphate and 10 μg/ml each of aprotinin and leupeptin (RIPA-buffer). After lysis, DNA and cell debris were pelleted by centrifugation at 20 000 g for 15 min and the supernatants were added to 5× SDS-sample buffer and 5% β-mercaptoethanol. Proteins were separated by SDSPAGE, followed by electrophoretic transfer to PVDF membranes (Bio-Rad, München, Germany). Blots were incubated overnight at 4°C with the monoclonal anti-phosphotyrosine antibody 4G10 (UBI, NY, USA). Immunoblotts were developed by incubation with horseradish peroxidase-conjugated protein G (BioRad) and using the ECL chemiluminescent substrate kit (Amer sham, Braunschweig, Germany).

Fgr-, Hck-, PAK- and Syk-immunocomplex kinase assay

For determination of kinase-activity, 8×105 cells (Fgr, Hck, PAK) or 1.5×107 (Syk) were infected with the indicated bacterial strain, lysed in RIPA-buffer and Hck, Fgr or Syk were immunoprecipitated overnight at 4°C using 5 μg of each antibody/sample (Santa Cruz Inc., Santa Cruz, CA). Control immunoprecipitates were performed with 3 μg of irrelevant affinity purified rabbit immunoglobulins (anti-EGF; Oncogene Science). After addition of protein A/G-coupled agarose (Santa Cruz Inc.) incubation was continued for at least 60 min. Immunoprecipitates were washed fourfold in lysis buffer, twice in kinase buffer [25 mM HEPES (pH 7.0), 150 mM NaCl, 10 mM MnCl2, 1 mM Na3VO4, 5 mM DTT, 0.5% NP40] and resuspended in 40 μl kinase buffer. The reaction was started by addition of unlabeled ATP (10 μM) and [γ-32P]ATP (10 μCi/sample; 3000 Ci/mmol; NEN/DuPont, Bad-Homburg, Germany) and samples were incubated for 15 min at 30°C. The kinase reaction was terminated by adding 5 μl boiling 5× SDS sample buffer and β-mercaptoethanol.
Inhibition of small GTPases by antisense oligonucleotides

After 4 days of differentiation, JOSK-M cells were incubated for 48 h with phosphorothioate oligonucleotides in RPMI1640 supplemented with 5% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin.

JNK-immunocomplex kinase assay

To measure the activity of JNK, infected or control cells were lysed in RIPA-buffer containing 0.2% SDS. Lysates were centrifuged at 25 000 g for 20 min, and JNK was immunoprecipitated from the supernatants at 4°C for 4 h using affinity purified rabbit-anti-JNK antibodies (Santa Cruz). Immunocomplexes were immobilized on agarose coupled protein A/G for an additional 60 min at 4°C, washed twice in RIPA-buffer, twice in 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM CaCl₂, 0.7 mM MgCl₂, 0.8 mM MgSO₄, 1% NP-40 and 2 mM Na3VO4, once in 100 mM Tris (pH 7.5) with 0.5 M LiCl and finally twice in kinase buffer consisting of 12.5 mM MOPS (pH 7.5), 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM MgCl₂, 0.5 mM NaF, and 0.5 mM Na3VO4. After washing, the immunocomplexes were resuspended in 50 μl kinase buffer supplemented with 10 μCi/sample [32P]ATP (6000 Ci/mmol, Nu/Du/Point), 10 μM ATP and 1 μg/ml of an N-terminal GST-c-Jun (amino acids 1–79) fusion protein. The samples were incubated at 30°C for 15 min and the reaction was stopped by addition of 5 μl boiling 5×reducing SDS-sample buffer. Samples were separated by 12% SDS–PAGE and analysed as above.

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References


