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CD66-mediated phagocytosis of Opa_{52} *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 Opa_{52}-expressing gonococci on human neutrophils. Here we show that Opa_{52}-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 Opa_{52}, since piliated Opa^- gonococci, commensal *Neisseria cinerea* or *E.coli* do not stimulate this signalling pathway. Inhibition of Src-like kinases or Rac1 prevents the uptake of Opa_{52} bacteria, demonstrating the crucial role of this signalling cascade for the opsonin-independent, Opa_{52}/CD66-mediated phagocytosis of pathogenic *Neisseria*.

Keywords: gonococci/phagocytes/Rac1/signal transduction/Src-like kinases

Introduction

Gonorrhea, 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 gonococci. 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 Opa_{50} protein promotes bacterial invasion into epithelial cells, whereas Opa_{52} and Opa_{60} 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 Opa_{50} was shown to recognise heparan sulfate containing surface proteoglycan receptors on human epithelial cells (Chen *et al.*, 1995; van Putten and Paul, 1995), whereas Opa_{52} and Opa_{60} 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 carcino-embryonic 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 (Brummer *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 Opa_{52}-expressing *N.gonorrhoeae* strain MS11 (N309 Opa_{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_{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 *N.gonorrhoeae* in professional phagocytes as an essential step during the course of an infection.

Results

Neisseria gonorrhoeae producing Opa_{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_{52}-expressing *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^-) Opa_{52}-expressing strain N309. Since gonococcal Opa^- P^- 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^- P^+ variant of *N.gonorrhoeae* MS11 for comparison with N309 Opa_{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 *Neisseria cinerea* strain N340 was used as a control in order to investigate the specificity of the induction pattern for *N.gonorrhoeae*.

Incubation of *in vitro*-differentiated JOSK-M cells with N309 Opa_{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_{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_{52} was inhibited by preincubation of JOSK-M cells with the kinase inhibitors (Figure 1). To demonstrate the significance of Opa_{52} in the induction of tyrosine phosphorylation, the *N.cinerea* strain N340 or the N280 Opa^- P^+ variant were incubated with the phagocytic cells. Neither of these triggered a significant increase in cellular tyrosine phosphorylation (Figure 1) and phosphorylation levels of the proteins were comparable to zero time of Opa_{52}-stimulated cells.

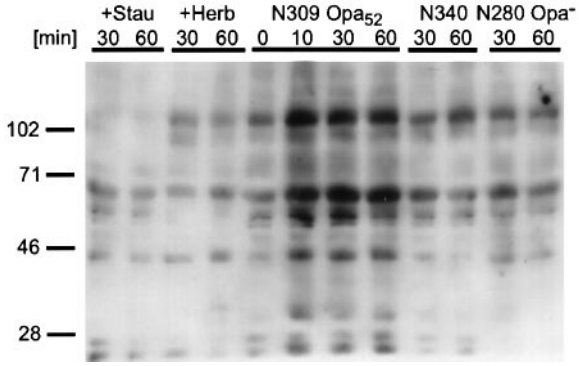

Fig. 1. Cellular tyrosine phosphorylation is induced by phagocytosis of *N.gonorrhoeae* N309 Opa_{52}. Increased tyrosine phosphorylation in JOSK-M cells upon infection with N309 Opa_{52} was already detected 10 min after the addition of bacteria and reached a maximum after 30 min. Preincubation of JOSK-M cells with herbimycin A (+Herb; 5 μM) or staurosporine (+Stau; 1 μM) reduced tyrosine phosphorylation of some proteins and completely abrogated the increase of tyrosine phosphorylation triggered by N309 Opa_{52}. The background phosphorylation of unstimulated cells is reflected by the zero time point of the infection with N309 Opa_{52} in the absence of inhibitors. Neither commensal *N.cinerea* N340 nor N280 Opa^- P^+ gonococci induced a significant change of tyrosine phosphorylation in JOSK-M cells. Whole cell lysates were separated by SDS–PAGE, blotted and analysed for tyrosine phosphorylation by incubation with the monoclonal anti-phosphotyrosine antibody 4G10. The figure shows a representative blot of three independent experiments.

Opsonin-independent internalisation of N309 Opa_{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_{52} *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_{52}, N280 Opa^- P^+, *N.cinerea* N340 or *Escherichia coli* H1887. Only N309 Opa_{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^- P^+, *N.cinerea* N340 or *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_{52} by *in vitro*-differentiated JOSK-M cells or primary neutrophils compared to N280 Opa^- P^+ and *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_{52}, demonstrating the pivotal role of the Opa_{52}/CD66 interaction for the uptake process. Inhibition of cellular kinases by staurosporine correlated with a complete blockade of the Opa_{52}-dependent phagocytosis of *N.gonorrhoeae*. Likewise, the Src-like tyrosine kinase inhibitor herbimycin A

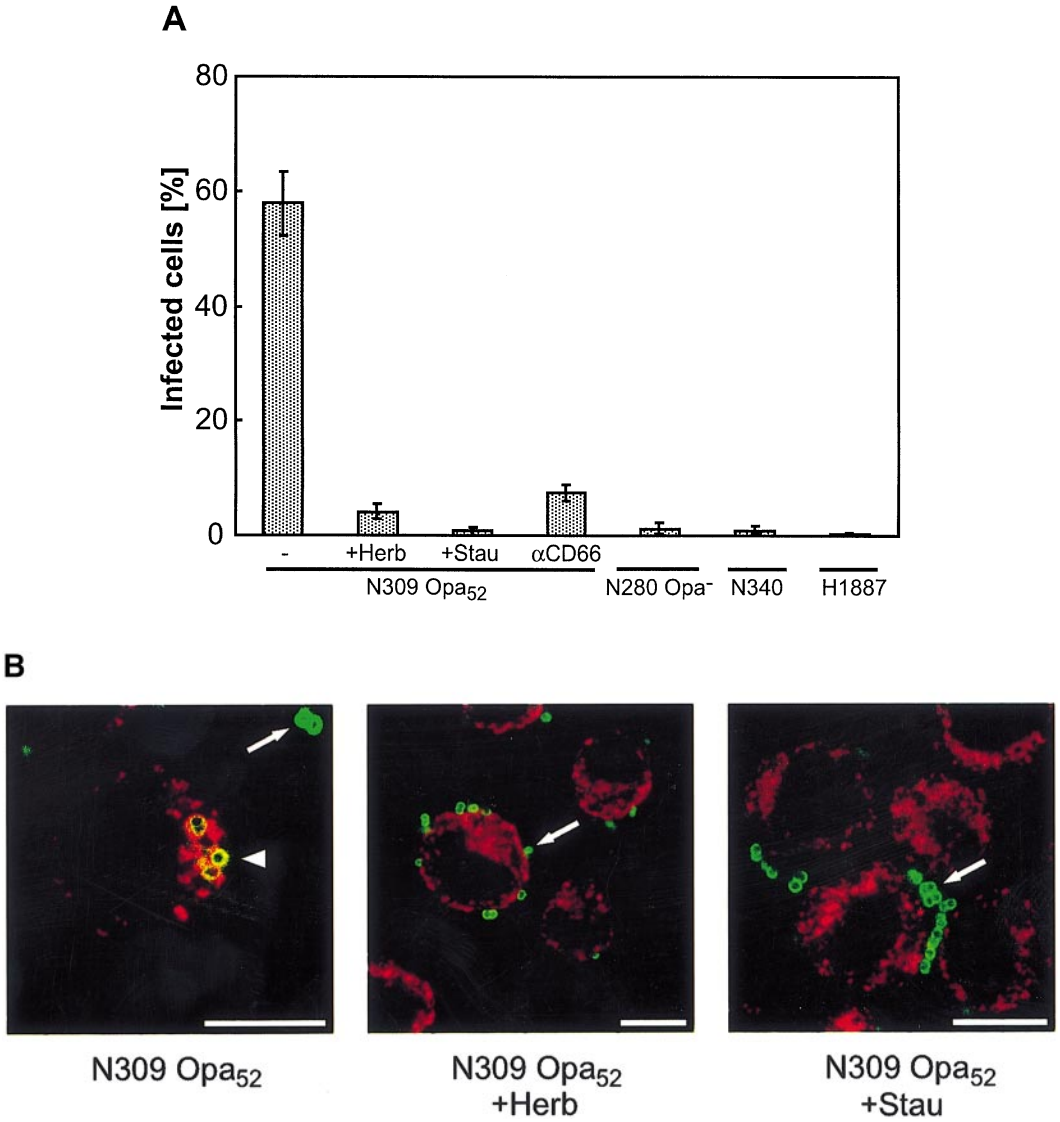

Fig. 2. Opsonin-independent phagocytosis of *N.gonorrhoeae* N309 Opa_{52} via CD66 depends on a herbimycin-A-sensitive mechanism. (**A**) N309 Opa_{52} was efficiently phagocytosed in the absence of opsonins, whereas N280 Opa^{-} P^{+}, *N.cinerea* N340 or *E.coli* H1887 were not taken up within the 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 Opa_{52}. 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 Opa_{52} *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 Opa_{52}, bacteria still adhered to the cells (arrows). Gonococci appear green and h-lamp-2 appears red in the pseudocolored images. Scale bars represent 10 μm.

almost completely abolished uptake of these bacteria (Figure 2A).

Phagocytosed N309 Opa_{52} 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 Opa_{52} to JOSK-M cells was not affected by either inhibitor.

Activation of Src-like protein tyrosine kinases in response to N309 Opa_{52}

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 Opa_{52} and the inhibition of the uptake of these bacteria by herbimycin A, only N309 Opa_{52} 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 Opa_{52}. Control immunoprecipitations from cells infected for 30 min with N309 Opa_{52} using irrelevant affinity purified rabbit antibodies demonstrated the specificity of the immunoprecipitation

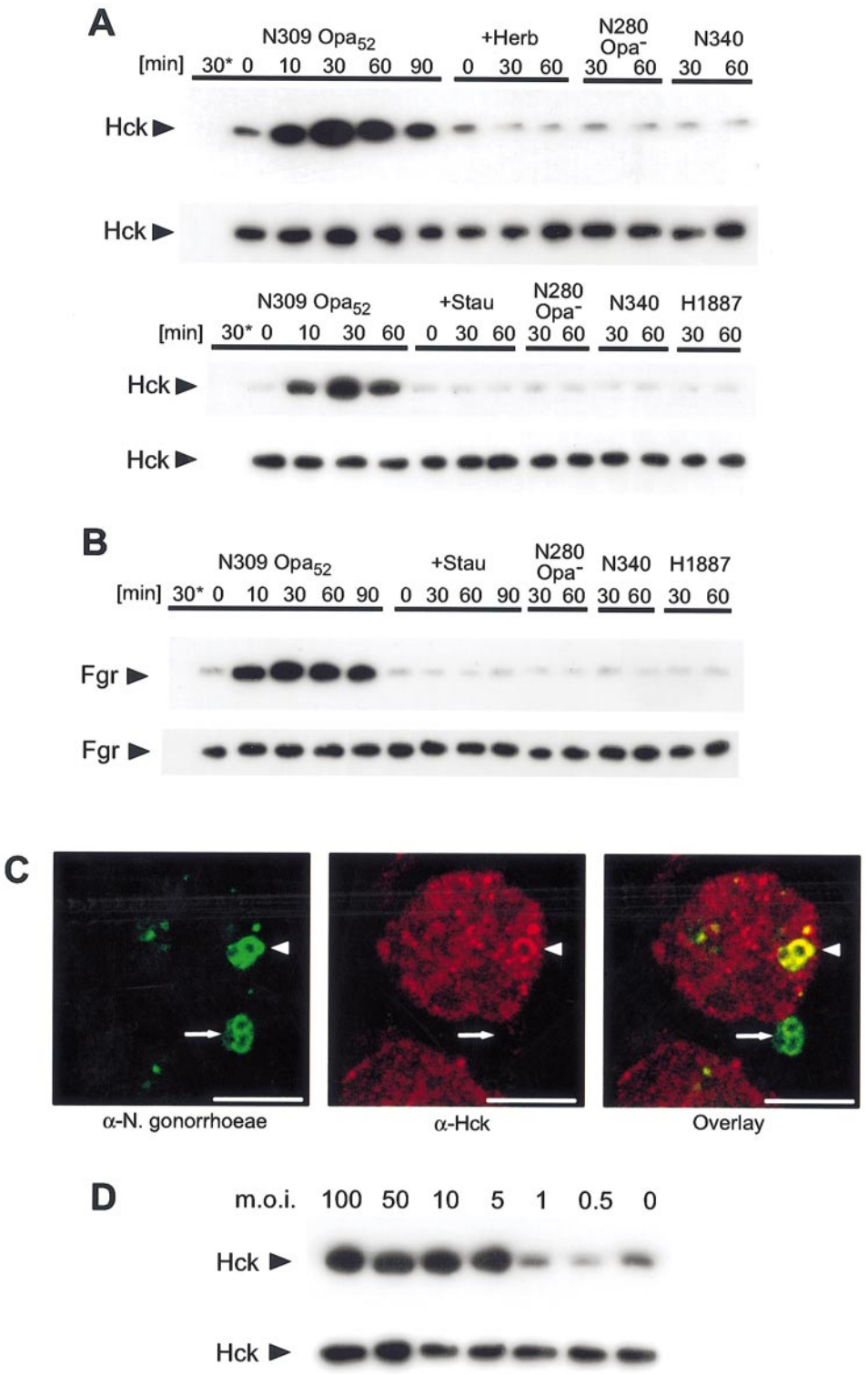

Fig. 3. The Src-like kinases Hck and Fgr are activated during phagocytosis of *N.gonorrhoeae* MS11 Opa_{52}. (A) and (B) Immunoprecipitation and immunocomplex kinase assays of Hck (**A**; upper panels) and Fgr (**B**; upper panel) revealed a strong induction of kinase activity during phagocytosis of N309 Opa_{52}. Preincubation with herbimycin A (+Herb; 5 μM) or staurosporine (+Stau; 1 μM) inhibited the activation of the kinases. Neither N280 Opa^{-} P^{+}, *N.cinerea* N340, nor *E.coli* H1887 stimulated kinase activity. Unspecific control immunoprecipitates (*) were performed from cells infected with N309 Opa_{52} 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 Opa_{52} induced a translocation of Hck to the phagosome. Cells were infected with N309 Opa_{52} 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 Opa_{52} 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).

(Figure 3A and B, see asterisk). The activation of Src-like kinases is also demonstrated by the recruitment of Hck to the *N.gonorrhoeae*-containing phagosome in JOSK-M cells (Figure 3C). Other Src-like kinases, in particular Fyn, were neither activated by infection of JOSK-M cells with N309 Opa_{52} nor with N280 $Opa^- P^+$ (not shown). Although in all of the experiments we used a multiplicity of infection (m.o.i.) of 50 bacteria/cell, significant activation of Hck was already detected at a m.o.i. of 5 (Figure 3D).

Involvement of the small GTP-binding protein Rac1 in the uptake of N309 Opa_{52}

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 Opa_{52}. Infection of JOSK-M cells with N309 Opa_{52} 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 $Opa^- P^+$ or *N.cinerea* N340 had no effect on Rac1-bound GTP level (Figure 4A). In contrast to Rac1, Cdc42Hs was not activated by N309 Opa_{52} (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 Opa_{52}, 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 Opa_{52}, 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 Opa_{52} 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 Opa_{52} 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 Opa_{52}, whereas N280 $Opa^- P^+$ or *N.cinerea* 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) (Coso *et al.*, 1995; Minden *et al.*, 1995). JNK was strongly stimulated upon infection of JOSK-M cells with N309 Opa_{52} (Figure 4E). Activation of JNK was restricted to infection by Opa_{52}-producing bacteria and was inhibited by pretreatment of the cells with herbimycin A (Figure 4E) or staurosporine (not shown).

Opa_{52}-induced stimulation of Src-like tyrosine kinases and JNK is mediated via CD66

It has been shown that Opa_{52} selectively binds to CD66 family members (Gray-Owen *et al.*, 1997). To further demonstrate the specificity of the observed signalling cascade for Opa_{52} proteins, we incubated phagocytic cells with recombinant *E.coli* DH5α producing Opa_{52} (H1907; Figure 5A). Activation of Hck or JNK was observed only after incubation of JOSK-M cells with H1907 Opa_{52}, whereas Opa-negative *E.coli (*H1887) failed to induce these activation events (Figure 5A and B), indicating a crucial role for the Opa_{52}/CD66 interaction in the observed signalling cascade.

To further demonstrate that the described pathway is distinct from Fcγ receptor-mediated cellular activation, we determined the activity of Syk, a tyrosine kinase known to be stimulated during Fcγ receptor-mediated uptake (Greenberg *et al.*, 1994). N309 Opa_{52} 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 Opa_{52}/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 Opa_{52}. 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 Opa_{52}

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

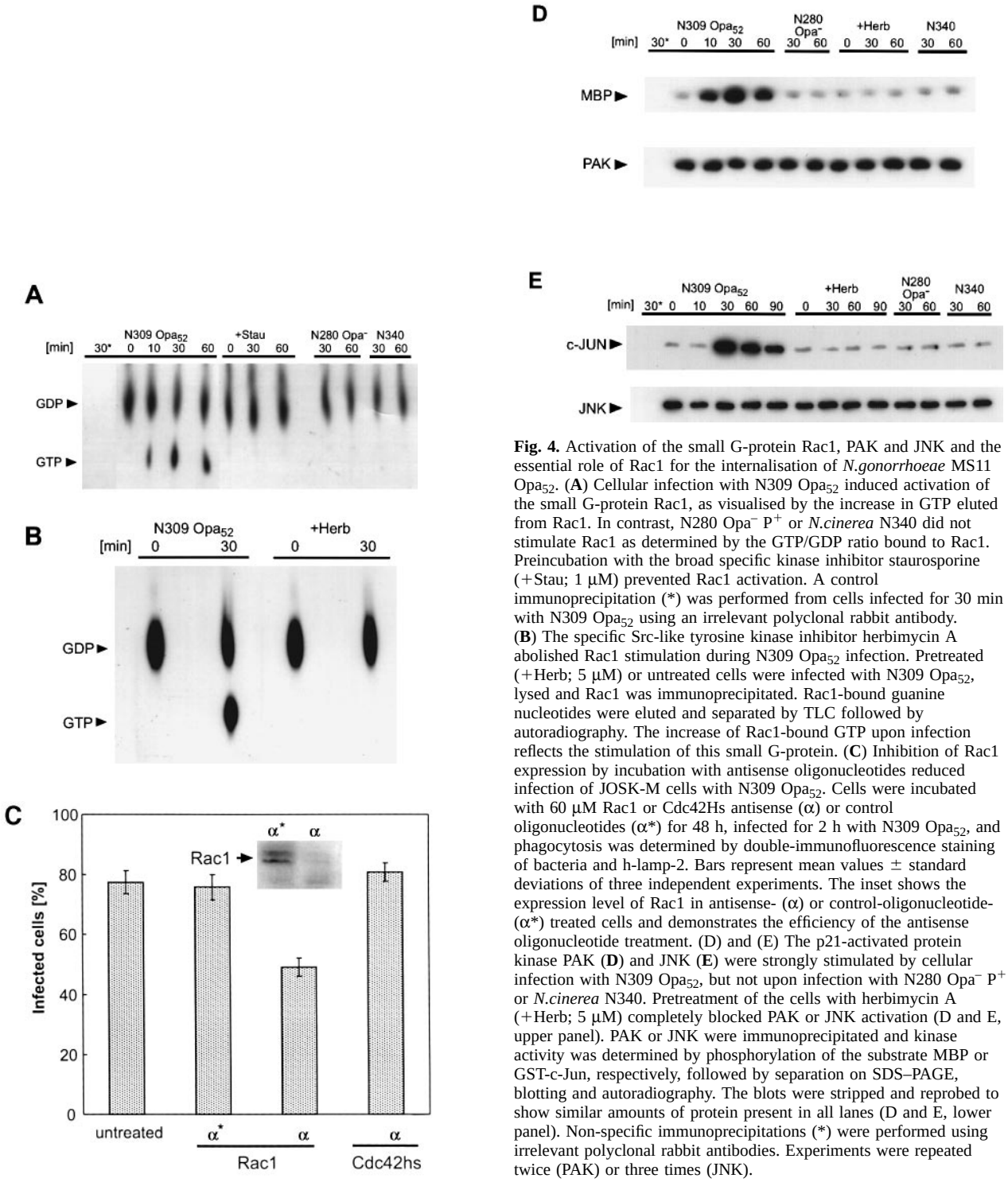

Fig. 4. Activation of the small G-protein Rac1, PAK and JNK and the essential role of Rac1 for the internalisation of *N.gonorrhoeae* MS11 Opa_{52}. (**A**) Cellular infection with N309 Opa_{52} induced activation of the small G-protein Rac1, as visualised by the increase in GTP eluted from Rac1. In 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 Opa_{52} using an irrelevant polyclonal rabbit antibody. (**B**) The specific Src-like tyrosine kinase inhibitor herbimycin A abolished Rac1 stimulation during N309 Opa_{52} infection. Pretreated (+Herb; 5 μM) or untreated cells were infected with N309 Opa_{52}, 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 Opa_{52}. Cells were incubated with 60 μM Rac1 or Cdc42Hs antisense (α) or control oligonucleotides (α*) for 48 h, infected for 2 h with N309 Opa_{52}, 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 Opa_{52}, 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).

human neutrophils infected with either N309 Opa_{52} or N280 Opa^{-} P^{+}. Fgr and JNK stimulation in primary neutrophils was similar to the activation observed in JOSK-M cells, thus indicating the biological relevance of the activation of Src-like protein tyrosine kinases and JNK by N309 Opa_{52} (Figure 6A and B).

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.*,

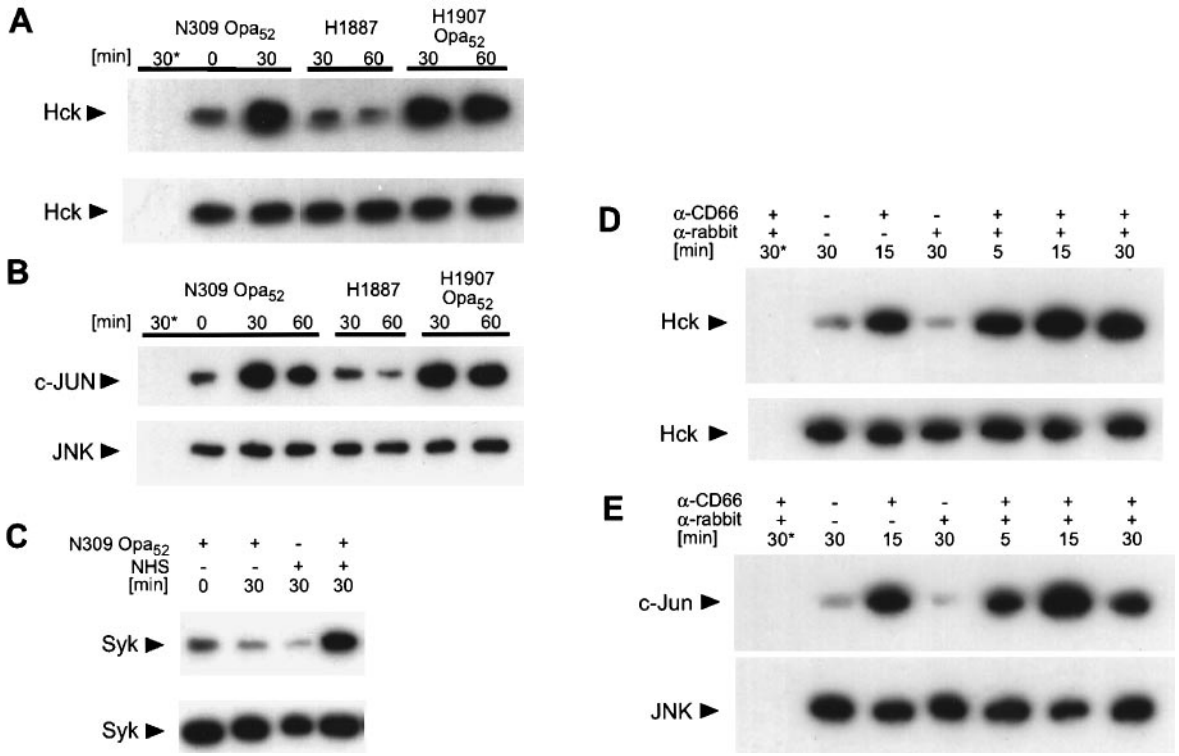

Fig. 5. Activation of Hck and JNK upon *N.gonorrhoeae* internalisation is mediated by Opa_{52} binding to CD66, independent of Syk activation, and mimicked by cellular triggering with anti-CD66 F(ab)-fragments. (**A**) Infection of JOSK-M cells with *E.coli* DH5α Opa_{52} (H1907) stimulated Hck to a similar extent as N309 Opa_{52}. *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 Opa_{52} was comparable to stimulation by N309 Opa_{52} (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 Opa_{52} resulted in activation of Syk. N309 Opa_{52} was opsonised by incubation with normal human serum (NHS) or left untreated. JOSK-M cells were stimulated with opsonised or unopsonised gonococci or with NHS alone and lysed after the indicated times. Syk was immunoprecipitated from post-centrifugation supernatants and activity of Syk was determined by autophosphorylation upon addition of $[^{32}P]\gamma ATP$ 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 Opa_{52} 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 Opa_{52} (N309) as well as *E.coli* DH5α Opa_{52} (H1907) by CD66 transfected HeLa cells (Gray-Owen *et al.*, 1997). In addition, phagocytosis is inhibited by anti-CD66 antibodies, indicating that Opa_{52}-mediated binding to CD66 is the trigger for the opsonin-independent uptake of these bacteria. Therefore, by using MS11 Opa_{52} or DH5α Opa_{52}, 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 Opa_{52}, 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 Opa_{52} 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 Opa_{52}/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

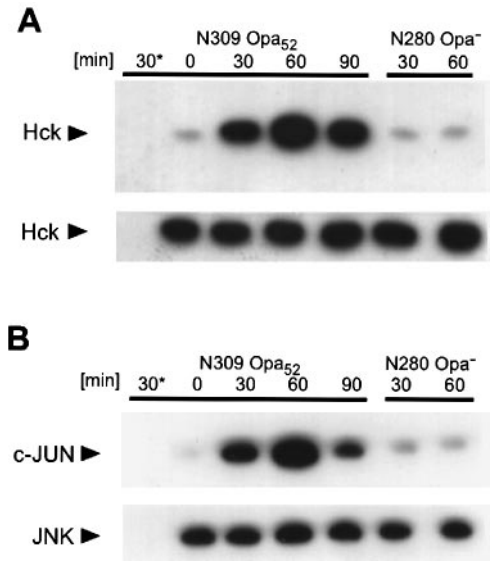

Fig. 6. Activation of Src-like kinases and JNK by *N.gonorrhoeae* N309 Opa_{52} in primary human polymorphonuclear granulocytes. PMN were obtained from healthy donors, infected with N309 Opa_{52} or N280 Opa^{-} P^{+} and activity of Hck (**A**) or JNK (**B**) was determined as described above (upper 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. Representative blots from two independent experiments are shown.

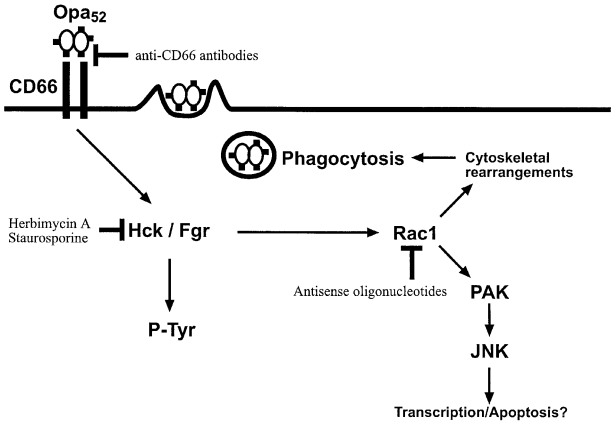

Fig. 7. Schematic representation of signalling events during opsonin-independent phagocytosis of *N.gonorrhoeae* N309 Opa_{52} via CD66. Internalisation of *N.gonorrhoeae* via binding of Opa_{52} to CD66 activates a signalling cascade from CD66 molecules via the Src-like tyrosine kinases Hck and Fgr to Rac1 and finally the downstream effector molecules PAK and JNK. Inhibition of either CD66-binding, Hck/Fgr or Rac1 prevents *N.gonorrhoeae* uptake showing the significance of the observed signalling cascade for internalisation of Opa_{52}-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.

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 Opa_{52}-dependent internalization, a tyrosine kinase that plays a key role in Fcγ receptor-mediated uptake of antibody-coated particles, supporting the view that phagocytosis via CD66 is distinct from opsonin-mediated uptake via Fcγ receptors. Whether Rac1 activation is specific for the opsonin-independent internalization remains to be determined.

The signal transduction cascade evoked by Opa_{52} gonococci is to some extent reminiscent of the signalling induced by binding or invasion of several pathogenic bacteria to human epithelial cells (Bliska *et al.*, 1993; Dehio *et al.*, 1995; Rosenshine *et al.*, 1996). *Salmonella typhimurium*, enteropathogenic *E.coli*, *Yersinia enterocolitica* and *Shigella flexneri* induce intensive tyrosine phosphorylation upon contact with their target cells. For example, *Y.enterocolitica* binds to β1 chain integrin receptors coupled to Src-like protein tyrosine kinase inducing tyrosine phosphorylation of several intracellular proteins (Isberg and Leong, 1990). Similarly, *S.flexneri* activates Src and mediates tyrosine phosphorylation of cortactin in epithelial cells (Dehio *et al.*, 1995). In the case of *Shigella* and *Yersinia*, tyrosine phosphorylation is a prerequisite for the invasion of the microorganisms into non-professional phagocytes. However, though there seem to be common themes in the signalling induced by these pathogens and gonococci, it is clear that the details of internalisation differ considerably between the different bacterial species, depending for example on the target cell type, the activation of distinct kinase subsets coupling to different pathways or the specific combination of certain signalling molecules.

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 Opa_{52} gonococci by phagocytes. Interestingly, whereas the human CD66 family members BGP and CGM1 possess tyrosine residues and SH2-domain-binding motives in their cytoplasmic domain, other members (CGM6, NCA) are GPI-linked membrane proteins (Thompson *et al.*, 1991). Both types of proteins are recognised by N309 Opa_{52} (Gray-Owen *et al.*, 1997) and both types could transduce the signal (Stevanova *et al.*, 1991; Brown, 1993) but their exact contribution to the observed signalling pathway has yet to be determined. In this respect, Opa_{52} gonococci could provide a highly selective and spatially localised trigger for future studies on transmembrane signalling via GPI-linked cell surface receptors and on the role that Src-like kinases play in these events.

Src-like tyrosine kinases are known to associate with cell surface receptors, e.g. the PDGF receptor or the T- or B-cell antigen receptors, and have been implicated in the transduction of signals from these receptors to small GTPases by phosphorylation of their guanine nucleotide exchange factors (Kypta *et al.*, 1990; Twamley-Stein *et al.*, 1993; Kefalas *et al.*, 1995; Xu *et al.*, 1996; Crespo *et al.*, 1997). Recently, Cdc42Hs and RhoA, two members of the Rho subfamily of Ras-related proteins, have been linked to the uptake of *S.typhimurium* and *S.flexneri*, respectively, by human epithelial cells (Adam *et al.*, 1996; Chen,L.-M. *et al.*, 1996; Watarai *et al.*, 1997). The activation of Rac1 by Opa_{52} gonococci may therefore

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 Opa_{52} 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 Opa^- *N.gonorrhoeae* MS11 expressed an Opa_{52}-homologue (Swanson *et al.*, 1988). Thus, expression of the Opa_{52} 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 opsonin-dependent mechanisms. The specific intracellular niche reached by gonococci 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 Opa_{52} 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 Opa_{52} 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 Opa_{52} 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 ACC30) and grown as suspension in RPMI1640 (Life Science Technologies) supplemented with 5% fetal calf serum (Boehringer Mannheim), 2 mM L-glutamine at 37°C, 5% CO_2. 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 CD66a, CD66b and CD66c.

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, herbimycin A (5 μM) for 12 h or polyclonal anti-CD66 antibodies (50 μg/ml; azide depleted; Dako, Hamburg, Germany) for 5 min prior to infection.

Bacteria

The gonococcal variants MS11-B1 (N273: $PilE_{B1}$ P^S) and MS11-F3 (N238: $PilE_{F3}$ 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 phagocyte-specific Opa protein (Opa_{52} 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% CO_2 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 Opa_{52} (H1907) and DH5α harbouring the empty expression vector pTrc99A (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, the optical density at 550 nm (gonococci) or 600 nm (*E.coli*) was determined 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 (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 *g* (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, Na_3VO_4 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 SDS–PAGE, 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). Immunoblots were developed by incubation with horseradish peroxidase-conjugated protein G (BioRad) and use of the ECL chemiluminescent substrate kit (Amersham, Braunschweig, Germany).

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

For determination of kinase-activity, 8×10^6 cells (Fgr, Hck, PAK) or 1.5×10^7 (Syk) were infected with the indicated bacterial strain, lysed in RIPA-buffer and Hck, Fgr or Syk were immunoprecipitated overnight at 4°C using 3 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 four times in lysis buffer, twice in kinase buffer [25 mM Hepes (pH 7.0), 150 mM NaCl, 10 mM $MnCl_2$, 1 mM Na_3VO_4, 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 [^{32}P]γATP (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

(5% final conc.). Samples were separated on SDS–PAGE, blotted and autoradiographies were performed. Aliquots of the immunoprecipitates were blotted with the immunoprecipitating antibody to analyse protein levels in all samples. Alternatively, blots were stripped for 45 min by incubation in 20 mM Tris (pH 6.8), 2% SDS, 70 mM β-mercaptoethanol at 70°C after primary analysis and reprobed to test for equal amounts of immunoprecipitated protein.

PAK kinase activity was determined by immunoprecipitation of PAK from stimulated or non-stimulated samples with 3 μg/sample affinity purified rabbit antibodies (Santa Cruz Inc., USA). The kinase buffer was supplemented with 3 mg/ml MBP (Sigma, Deisenhofen, Germany) and the activity of PAK was determined by incorporation of [^{32}P] into the substrate MBP for 20 min at 30°C. Samples were separated by SDS–PAGE and analysed as above.

Immunofluorescence staining and determination of infected cells

After 60 min of infection, cells were centrifuged onto glass coverslips in 24-well plates (Nunc, Roskilde, Denmark) and fixed for 20 min in 3% paraformaldehyde in PBS at room temperature. After three washes with PBS, samples were incubated for 5 min in the same buffer supplemented with 10% fetal calf serum and 0.2% saponin in order to block unspecific binding sites and permeabilise the cells. Suitable dilutions of polyclonal rabbit anti-*N.gonorrhoeae* MS11 (AK92), polyclonal rabbit anti-*N.cinerea* (AK180), or polyclonal rabbit-anti-*E.coli* OmpA (generous gift from Y.-D.Stierhof, MPI für Biologie, Tübingen, Germany) and monoclonal mouse-anti-human lysosome-associated membrane protein 2 (h-lamp-2; clone H4B4; DSHB, University of Iowa), or monoclonal mouse anti-*N.gonorrhoeae* MS11 (AK135) and polyclonal affinity purified rabbit-anti-Hck (Santa Cruz Inc., USA), respectively, in PBS with 10% fetal calf serum and 0.2% saponin were applied to the samples for 1 h at room temperature. Cells were washed twice with PBS, blocked again for 5 min and incubated with FITC-conjugated goat-anti-rabbit and Texas Red-conjugated goat-anti-mouse-antibodies or Texas Red-conjugated goat-anti-rabbit and FITC-conjugated goat-anti-mouse, respectively, for 45 min at room temperature (all goat antibodies were purchased from Dianova, Hamburg, Germany). After three washes, coverslips were mounted in glycerol medium, sealed with nail polish, and viewed with a Leica TCS 4D confocal laser scanning microscope equipped with an argon/krypton mixed gas laser (Leica Lasertechnik, Heidelberg, Germany). Images were taken serially using appropriate excitation and emission filters for the fluorescent dyes. The corresponding images were digitally processed with Photoshop 3.0 (Adobe Systems, Mountain View, CA) and merged to yield pseudocolored RGB pictures. Intracellular bacteria were detected by their co-localisation with the phagosomal/lysosomal membrane protein h-lamp-2 and the percentage of infected cells was determined by counting at least 200 cells in each of three independent infections.

Determination of Rac1 and Cdc42Hs activation state

JOSK-M cells were washed twice in phosphate-free DMEM-medium, resuspended in the same medium complemented with 10% dialysed fetal calf serum and labeled for 4 h with 1 mCi/ml [$^{32}P_i$]. Prior to infection, cells were treated for 8 h with 5 μM herbimycin A or for 10 min with 1 μM staurosporine, and these inhibitors were maintained throughout the metabolic labelling period. Infection was terminated by lysis in 25 mM Tris–HCl (pH 7.5), 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 20 mM $MgCl_2$, 450 mM NaCl and 10 μg/ml each of aprotinin and leupeptin (lysis buffer). Nuclei and cell debris were removed by centrifugation (20 000 *g*) at 4°C for 20 min. Rac1 or Cdc42Hs were immunoprecipitated from the lysates at 4°C for 45 min using polyclonal, affinity purified anti-Rac1 or anti-Cdc42Hs-antibodies, respectively (Santa Cruz Inc.). The immunoprecipitates were collected by further incubation with protein A/G-conjugated agarose beads (Santa Cruz Inc.). Precipitates were washed seven times in lysis buffer, once in 25 mM Tris–HCl (pH 7.5), 0.1% Triton X-100, 1 mM $MgCl_2$ and 125 mM NaCl and bound nucleotides were eluted in 1 mM EDTA at 68°C for 20 min. The samples were then centrifuged at 15 000 *g* for 5 min and nucleotides in the supernatant were separated on PEI-cellulose plates (Machery & Nagel, Düren, Germany) with 0.75 M KH_2PO_4 (pH 3.5). The TLC plates were analysed by autoradiography. Activation of Rac1 is detected by an increase in the GTP/(GDP + GTP) ratio.

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. ACTTGATGGCCTGCA complementary to the 5′-end of Rac1 mRNA, TGGCTATGCCACATG, a Rac1 control oligonucleotide containing the same bases in a randomised order, ACTTAATTGTCTGCA complementary to the 5′-end of Cdc42Hs mRNA and TGACTATTCCATATG composed of the same nucleotides in a randomised order (B&G Biotech, Denzlingen, Germany) were added to the cells at a final concentration of 10 or 60 μM, respectively. After 48 h, the cells were washed twice in RPMI1640 containing 5% FCS and either infected with MS11 Opa_{52} at a m.o.i. of 50 for 2 h or lysed without infection for analysis of Rac1 and Cdc42Hs expression. After infection, the cells were washed three times with PBS and centrifuged on coverslips in 24-well plates prior to fixation with 3% paraformaldehyde. Preparations were stained with antibodies against gonococci and h-lamp-2 and the percentage of cells with internalised gonococci was determined.

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-human 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 $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$, 1% NP-40 and 2 mM Na_3V0_4, 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 $MgCl_2$, 0.5 mM NaF, and 0.5 mM Na_3VO_4. After washing, the immunoprecipitates were resuspended in 30 μl kinase buffer supplemented with 10 μCi/sample [^{32}P]γATP (6000 Ci/mmol, NEN/DuPont), 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 10% SDS–PAGE and analysed by autoradiography. The kinase substrate GST-c-Jun was expressed in *E.coli* DH5α after transformation, growth for 12 h and incubation with IPTG (200 μM) for 4 h. Bacteria were lysed in 25 mM HEPES (pH 7.4), 0.2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM NaCl and 10 μg/ml each of aprotinin and leupeptin; GST-fusion proteins were purified by binding to glutathione agarose and eluted in kinase buffer with 20 mM glutathione. Purity of the preparations was tested by SDS–PAGE and Coomassie-staining.

Activation of CD66 by anti-CD66 antibodies

Anti-CD66-F(ab)-fragments were prepared by incubation of 100 μl antibodies (1 mg/ml; Dako, Hamburg, Germany) with 1 U papain immobilized on agarose-beads (Sigma, Deisenhofen, Germany) at 37°C for 4 h. Papain-agarose was removed by repeated centrifugation in a microcentrifuge at 14 000 r.p.m. Protein A/G-plus (Santa Cruz Inc.) was added to the supernatants and samples were incubated overnight at 4°C. Fc-fragments were removed by repeated centrifugation at 14 000 r.p.m. Twenty-five μl of anti-CD66-F(ab)-fragment were applied for 15 min on ice to 8×10^6 JOSK-M cells suspended in 2 ml RPMI1640, 5% FCS. Samples were transferred to 37°C and 25 μl of mouse-anti-rabbit-$F(ab)_2$-fragments (Sigma Immunochemicals) per sample were added for the indicated time. Cells were pelleted by centrifugation at 250 *g* for 2 min at 4°C and lysed in RIPA buffer for immunoprecipitation and kinase assays.

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