

# Granulocyte CEACAM3 Is a Phagocytic Receptor of the Innate Immune System that Mediates Recognition and Elimination of Human-specific Pathogens

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## Abstract

Carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) are used by several human pathogens to anchor themselves to or invade host cells. Interestingly, human granulocytes express a specific isoform, CEACAM3, that participates together with CEACAM1 and CEACAM6 in the recognition of CEACAM-binding microorganisms. Here we show that CEACAM3 can direct efficient, opsonin-independent phagocytosis of CEACAM-binding *Neisseria*, *Moraxella*, and *Haemophilus* species. CEACAM3- but not CEACAM6-mediated uptake is blocked by dominant-negative versions of the small GTPase Rac. Moreover, CEACAM3 engagement triggers membrane recruitment and increased GTP loading of Rac that are not observed upon bacterial binding to CEACAM6. Internalization and Rac stimulation are also inhibited by compromising the integrity of an immunoreceptor tyrosine-based activation motif (ITAM)-like sequence in the cytoplasmic tail of CEACAM3 or by interference with Src family protein tyrosine kinases that phosphorylate CEACAM3. In contrast to interfering with CEACAM6, blockage of CEACAM3-mediated events reduces the ability of primary human granulocytes to internalize and eliminate CEACAM-binding bacteria, indicating an important role of CEACAM3 in the control of human-specific pathogens by the innate immune system.

Key words: *Neisseria gonorrhoeae* • Opa protein • *Haemophilus influenzae* • *Moraxella catarrhalis* • innate immunity

## Introduction

The gram-negative pathogens *Neisseria gonorrhoeae*, *N. meningitidis*, *Moraxella catarrhalis*, and *Haemophilus influenzae* are highly adapted to the human as their sole natural host. They colonize mucosal surfaces in different parts of the body: the nasopharynx in the case of *N. meningitidis*, *M. catarrhalis*, and *H. influenzae* or the urogenital tract in the case of *N. gonorrhoeae*. Though colonization with these pathogens can develop into invasive and sometimes life-threatening diseases, colonization most often (*N. meningitidis*, *M. catarrhalis*, and *H. influenzae*) or in a large proportion of the cases (*N. gonorrhoeae*) remains without symptoms (1). In particular, in gonorrhea the often mild symptoms can resolve despite the lack of effective acquired immunity (1–3), suggesting that innate immune defenses are able to contain the pathogen.

The four pathogenic species share several common adaptations such as phase or antigenic variation of surface structures and expression of outer membrane proteins that mediate the binding to members of the carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family on human cells (4–8). In the case of *H. influenzae*, CEACAM recognition is mediated by the P5 protein found on typeable and non-typeable strains (9), whereas UspA1 has been identified recently as the *M. catarrhalis* CEACAM-binding protein (7). CEACAM recognition by pathogenic *Neisseriae* is mediated by members of the phase-variable Opa protein family (Opa<sub>CEA</sub>) (for review see reference 10).

Opa<sub>CEA</sub> proteins from gonococci and meningococci can recognize four members of the human CEACAM family, namely CEACAM1, CEACAM3, CEA, and CEACAM6 (6, 11, 12). The complete spectra of CEACAMs recog-

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Abbreviations used in this paper: CEACAM, carcinoembryonic antigen-related cell adhesion molecule; ITAM, immunoreceptor tyrosine-based activation motif; PI-3K, phosphatidylinositol 3'-kinase; PTK, protein tyrosine kinase.

nized by the P5 protein of *H. influenzae* or the UspA1 protein of *M. catarrhalis* have not been analyzed in detail, but they seem to overlap the spectrum of Opa<sub>CEA</sub>-recognized CEACAMs (7, 8). Except for CEACAM1, homologues of these human receptors have not been detected in rodents, limiting the in vivo analysis of CEACAM function.

In vitro experiments suggest that several CEACAMs contribute to cell–cell adhesion by homo- or heterotypic interaction and participate in signal transduction into the cell (13). With regard to pathogen-induced signaling events, Opa<sub>CEA</sub>-mediated engagement of CEACAM1 expressed on activated CD4-positive T cells has been shown to cause an arrest of T cell proliferation in vitro (14). This immunosuppressive effect is presumably due to negative signals emanating from a functional immunoreceptor tyrosine-based inhibitory motif in the cytoplasmic tail of CEACAM1 (15). CEACAM1 together with CEACAM3 and CEACAM6 is expressed on granulocytes that internalize CEACAM-binding bacteria in the absence of opsonizing antibodies or complement factors (4, 5, 16, 17). This opsonin-independent phagocytosis is accompanied by increased activity of Src family protein tyrosine kinases (PTKs), stimulation of the small GTPase Rac, and prominent rearrangements of the actin cytoskeleton (17, 18), suggesting that human phagocytes can specifically detect and efficiently internalize bacteria that express CEACAM-binding adhesins. However, it is so far unclear which of the different CEACAM receptors expressed on granulocytes is responsible for the signaling events observed in response to CEACAM-binding pathogens and if these biochemical events are linked to the elimination of CEACAM-binding bacteria by human phagocytes.

In this study, we demonstrate that the granulocyte-specific orphan receptor CEACAM3 is linked to the stimulation of the small GTPase Rac in response to pathogenic, CEACAM-binding *N. gonorrhoeae*, *M. catarrhalis*, and *H. influenzae*. Stimulation of Rac is dependent on Src family kinase-mediated phosphorylation of the ITAM-like sequence in the cytoplasmic domain of CEACAM3 and leads to the efficient internalization of the pathogens. Since blockage of CEACAM3 or interference with Rac stimulation reduce phagocyte clearance of CEACAM-binding bacteria, our results suggest an important function for CEACAM3 in the control of human-specific pathogens by the innate immune system.

## Materials and Methods

**Bacteria.** Opa<sub>CEA</sub>-expressing (Opa<sub>52</sub>), nonpiliated *N. gonorrhoeae* MS11-B2.1 (strain N309), nonopaque, piliated gonococci MS11, and commensal *N. cinerea* were provided by Thomas Meyer (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany). *Neisseria* were grown on GC-agar (GIBCO BRL) supplemented with vitamins at 37°C, 5% CO<sub>2</sub> and subcultured daily. The unencapsulated variant of *H. influenzae* strain RD (obtained from A. Reidl, Zentrum für Infektionsforschung) and *M. catarrhalis* strain 11994 (obtained from DSMZ) were grown on BHI agar at 37°C, 5% CO<sub>2</sub>. For certain assays, bacteria were labeled with

0.2 µg/ml 5-(6)-carboxyfluorescein-succinylester (FITC; Molecular Probes) in PBS for 15 min at 37°C in the dark and washed three times with PBS before use.

**Cell Culture.** The human embryonic kidney cell line 293T (293 cells) was grown in DMEM/10% calf serum at 37°C, 5% CO<sub>2</sub>. Cells were subcultured every 3–4 d. Prior to infection and Rac pull-down assays, cells were serum starved overnight in DMEM containing 0.5% calf serum. Primary human granulocytes were purified from freshly drawn blood as described (17). Viability of cells was determined before infection using trypan blue staining and in all cases was >90%.

**Recombinant DNA Constructs.** Plasmids encoding cDNAs of human CEACAM1, CEACAM3, CEACAM3 Y241F, and CEACAM6 were provided by Wolfgang Zimmermann (Universitätsklinikum Grosshadern, München, Germany). CEACAM3 WT and CEACAM3 Y241F were amplified with primers CEA3HA-sense, 5'-GGGAAGCTTGCCATGGGGCCCCCCTCAGCCTCTCCCCAC-3' and CEA3HA-anti, 5'-GGGGACGTCATAGGGATAAGAAGCCACTTCTGCTTTGTGGTC-CATCCG-3' and subcloned into the HindIII-AatII-digested plasmid pBluescript FAK-HA (19) allowing an in-frame fusion of the COOH-terminal cytoplasmic domain of CEACAM3 with a double HA tag. CEACAM3 lacking the cytoplasmic domain (CEACAM3 ΔCT) was constructed accordingly using primers CEA3HA-sense and CEA3HA-deltaCTanti, 5'-GGGGACGTCATAGGGATATTTGGCAAGGAGCAGGAAACACACCA-GTGC-3'. Introduction of the Y230F point mutation into CEACAM3 WT and CEACAM3 Y241F was accomplished using the Quikchange mutagenesis kit (Stratagene), resulting in CEACAM3 Y230F and CEACAM3 Y230/241F. CEACAM3 constructs were verified by sequencing and subcloned via HindIII-NotI restriction sites into pcDNA3.1 (Invitrogen). Myc-tagged Rac N17 and Cdc42 N17 were provided by Alan Hall (University College, London, UK), VSV-tagged Rho N19 was provided by Thomas Adam (Universitätsklinikum Charité, Berlin, Germany), kinase-inactive c-Src (Src K297M) was provided by David Schlaepfer (The Scripps Research Institute, La Jolla, CA), and the GST-PAK CRIB domain (GST-CRIB) was provided by Gary Bokoch (The Scripps Research Institute, La Jolla, CA). GST-CRIB was expressed in *Escherichia coli* BL-21 and purified using GSTrap FF (Amersham Biosciences). TAT-RacN17, TAT Cdc42N17, and TAT-LacZ constructs were provided by Steve Dowdy (University of California at San Diego, La Jolla, CA), and the encoded proteins were expressed and purified as described (20).

**Reagents and Antibodies.** Phalloidin-TRITC was from Molecular Probes. Rabbit antiserum against *N. gonorrhoeae* (AK92) was provided by Thomas Meyer, and mAbs against the Opa proteins (clone 4B12) were from Mark Achtman (Max-Planck-Institut für Infektionsbiologie, Berlin, Germany), against the VSV epitope (clone P5D4) were from Sigma-Aldrich, against the myc-epitope (clone 9E10) were from Santa Cruz Biotechnology, against Rac (clone 23A8) were from Upstate Biotechnology, and against endoglin (clone P3D1) were from the Developmental Studies Hybridoma Bank. mAb against the HA tag (clone 12CA5) and against Src (clone 2-17) were purified from hybridoma supernatants. mAb Kat4c recognizing CEACAM1, CEA, and CEACAM6 was from DAKO, mAb COL-1 reacting with CEACAM3 and CEA was purchased from Zymed Laboratories, mAbs 26/3/13, specific for CEA, 9A6, specific for CEACAM6, and D14HD11, crossreactive with all CEACAMs, were from Genovac. Secondary antibodies were from Jackson ImmunoResearch Laboratories. Prior to phagocytosis inhibition experiments,

antibodies were cleared from sodium azide by 10 washes with PBS using Vivaspin concentrators 50 K (Vivascience).

**Electron Microscopy.** Phagocytes were seeded at  $5 \times 10^4$  cells/well in 24-well plates on acid-washed glass coverslips and infected with 10 bacteria per cell. After 1 h, infected cells were fixed with 2% glutaraldehyde in PBS for at least 1 h at 4°C and postfixed with 1% osmium tetroxide in PBS for 1 h on ice. The samples were dehydrated in a graded series of ethanol, critical point dried from CO<sub>2</sub>, sputter coated with 8 nm gold-palladium, and examined at 25 kV accelerating voltage in a Hitachi S-800 field emission scanning electron microscope.

**Transfection of Cells, Cell Lysis, and Western Blotting.** 293 cells were transfected by the standard calcium phosphate coprecipitation method using 5 µg of CEACAM constructs or empty control vector. For cotransfection, 5 µg of cotransfected constructs together with 3 µg of CEACAM constructs, respectively, were used, and in all samples total DNA was adjusted to 8 µg using the empty control vector. Cells were employed in infection experiments 48 h after the transfection. Cell lysis and Western blotting was performed as described (21).

**Immunofluorescence Staining.** Immunofluorescence labeling was performed as described (17). For differentiating between extra- and intracellular bacteria, cells were infected with FITC-labeled bacteria, and fixed samples were stained before permeabilization with polyclonal rabbit-anti-*N. gonorrhoeae* and goat-anti-rabbit-Cy5 in staining buffer (PBS, 5% FCS), resulting in FITC-labeled intracellular and FITC/Cy5-labeled extracellular bacteria. After permeabilization of the cells, phalloidin-TRITC was used to stain cellular actin.

**Gentamicin Protection Assay.** For gentamicin protection assays,  $6 \times 10^5$  293 cells in 24-well plates were infected with 20 bacteria/cell. After the infection, the medium was replaced with DMEM containing 50 µg/ml gentamicin. After 45 min of incubation, cells were lysed by the addition of 1% saponin in PBS for 20 min. Suitable dilutions were plated to determine the number of recovered viable bacteria.

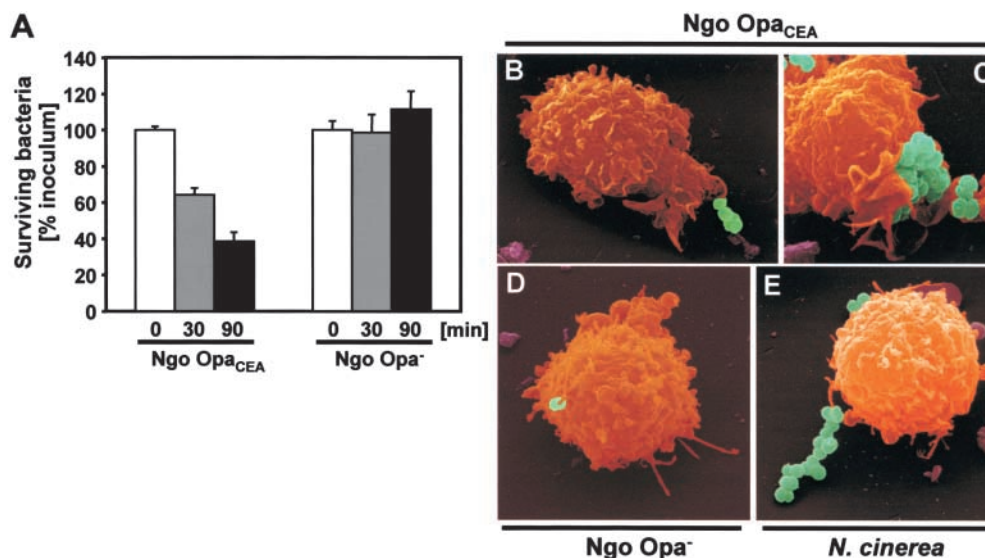
**Determination of Rac-GTP Loading.** Rac-GTP loading in transfected 293 cells was determined using a GST fusion protein encompassing the CRIB domain of PAK (22). Briefly, cells were

lysed in Triton buffer (25 mM Hepes, pH 7.4, 1% Triton X-100, 150 mM NaCl, 20 mM MgCl<sub>2</sub>, 10% glycerol, 10 mM sodium pyrophosphate, 100 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, and 10 µg/ml each of aprotinin, leupeptin, pefabloc, and pepstatin), and cleared lysates were incubated with 10 µg of GST-CRIB immobilized on glutathione-agarose beads for 45 min at 4°C. After three washes with Triton buffer, the precipitates were taken up in 2× SDS sample buffer and analyzed by Western blotting.

**Granulocyte Phagocytosis.** Phagocytosis was determined by FACS® analysis according to Voyich et al. (23). Briefly, 10<sup>6</sup> granulocytes were infected with  $2 \times 10^7$  FITC-labeled bacteria in 1 ml phagocytosis buffer (PB; 1× PBS, 10 mM glucose, 1% heat inactivated serum) for 15 min at 37°C. In inhibition experiments, 20 µg of the indicated, azide-free antibodies were added to the cells 1 min before the infection. Phagocytosis was stopped by addition of ice-cold PB, samples were washed, taken up in cold PBS, 2% FCS, 2 mg/ml Trypan blue, and analyzed on a FACS-Calibur (Becton Dickinson). To obtain an estimate of the amount of phagocytosed bacteria, the percentage of FITC-positive granulocytes was multiplied by the mean fluorescence of these cells. For TAT-mediated protein transduction, granulocytes were incubated for 1 h at 37°C with the indicated amounts of purified TAT fusion proteins before infection and analyzed as above. In control experiments, granulocytes were incubated with FITC-labeled TAT-RacN17 and observed by confocal microscopy to confirm the intracellular localization of the protein (Fig. S1 C, available at <http://www.jem.org/cgi/content/full/jem.20030204/DC1>).

**Phagocyte-mediated Bacterial Killing.** Assays were performed according to Virji et al. (24). Briefly,  $2 \times 10^6$  bacteria were added to 10<sup>6</sup> PMN in 300 µl DMEM, 0.5% heat-inactivated FCS in 24-well plates. Before and after a 60-min incubation at 37°C, aliquots of the samples were collected and dilutions plated on GC agar plates to determine the number of viable bacteria. In inhibition experiments, 20 µg of the indicated, azide-free antibodies were added to the cells 1 min before the infection.

**Online Supplemental Material.** Fig. S1 demonstrates (A) quenching of the fluorescence emitted by FITC-labeled bacteria upon Trypan blue addition; (B) Expression and purification of TAT-



**Figure 1.** OpacEA-expressing *N. gonorrhoeae* are efficiently recognized and eliminated by human granulocytes via lamellipodia-like membrane protrusions. (A) Granulocytes were incubated with OpacEA gonococci (Ngo OpacEA) or nonopaque, piliated gonococci (Ngo Opa<sup>-</sup>), respectively. At the indicated times, the number of viable gonococci was determined and expressed as the percentage of the initial inoculum. The graph shows mean values ± SDs of a representative experiment done in triplicate. Equivalent results were obtained with granulocytes isolated from four different donors. (B–E) Pseudocolored scanning electron micrographs of granulocytes infected for 1 h with OpacEA gonococci (B and C), nonopaque *N. gonorrhoeae* (D), or nonpathogenic *N. cinerea* (E).

fusion proteins; and (C) TAT-mediated protein transduction of human granulocytes. Fig. S2 shows (A) FACS<sup>®</sup> analysis of CEACAM expression on primary human granulocytes; (B) Oxidative response of granulocytes upon infection with gonococci; (C and D) CEACAM expression by transiently transfected 293 cells analyzed by Western blotting (C) or FACS<sup>®</sup> (D), respectively; and (E) adhesion of Opa<sub>CEA</sub>-expressing *N. gonorrhoeae* to CEACAM-transfected 293 cells as observed by immunofluorescence microscopy. Figs. S1 and S2 are available at <http://www.jem.org/cgi/content/full/jem.20030204/DC1>.

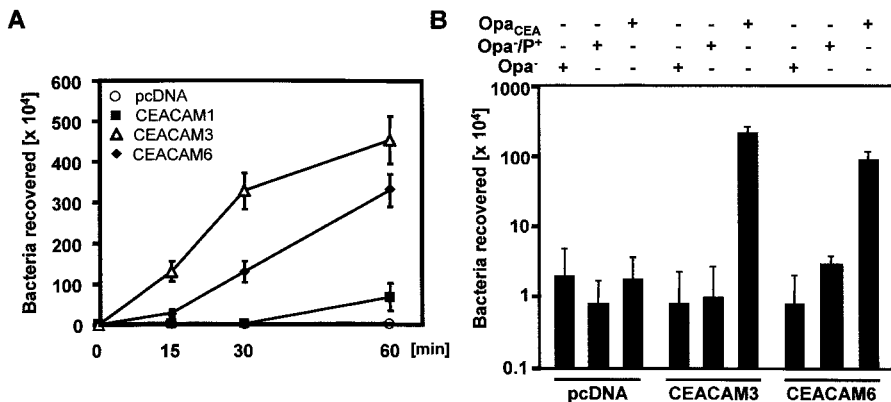
## Results

**Human Granulocytes Eliminate Opa<sub>CEA</sub>-expressing Gonococci.** Human granulocytes express three of the four CEACAM family members, namely CEACAM1, CEACAM3, and CEACAM6, that bind neisserial Opa<sub>CEA</sub> proteins (Fig. S2 A, available at <http://www.jem.org/cgi/content/full/jem.20030204/DC1>). Accordingly, primary granulocytes can efficiently recognize and eliminate Opa<sub>CEA</sub> *N. gonorrhoeae* in the absence of specific antibodies or complement (4, 16). Indeed, when Opa<sub>CEA</sub> gonococci were incubated with granulocytes at a 2:1 ratio, only ~40% of the initial bacterial inoculum could be recovered after 90 min (Fig. 1 A). Nonopaque organisms were not recognized or eliminated (Fig. 1 A). Contact of granulocytes with Opa<sub>CEA</sub> *N. gonorrhoeae* stimulated the cells to produce reactive oxygen derivatives (Fig. S2 B) and to exhibit pronounced lamellipodia-like protrusions on the surface (Fig. 1, B and C). These membrane protrusions were involved in the engulfment of the bacteria (Fig. 1 C). In contrast, nonopaque gonococci only moderately stimulated the oxidative burst of the granulocytes (Fig. S2 B), and neither nonopaque gonococci nor nonpathogenic *N. cinerea* induced lamellipodia-like protrusions on the phagocyte surface, indicating that the observed responses were due to the Opa<sub>CEA</sub>-CEACAM interaction (Fig. 1, D and E). These results suggested that granulocytes express CEACAM family member(s) with phagocytic properties. This receptor(s) seems to transduce signals that stimulate bactericidal mechanisms resulting in elimination of CEACAM-binding bacteria. However, the identity of the

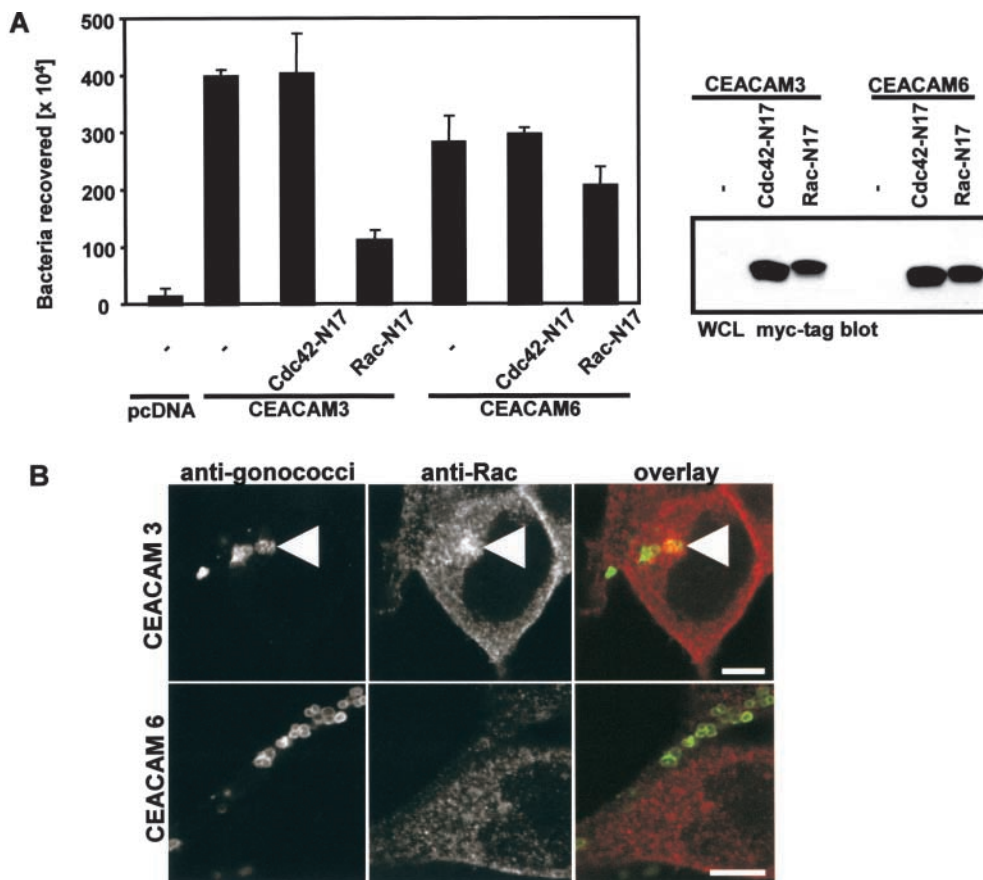
CEACAM family member(s) responsible for these processes was unknown.

**CEACAM3 and CEACAM6 Mediate Efficient Uptake of Opa<sub>CEA</sub>-expressing Bacteria.** To study the contribution of specific CEACAM molecules to the internalization of CEACAM-binding bacteria, we took advantage of 293 cells that do not express members of the CEACAM family (Fig. S2, C and D; reference 25). Transfection of 293 cells with CEACAM1, CEACAM3, CEACAM6, or the empty control vector (pcDNA) resulted in the surface expression of the respective receptor in ~70–80% of the cell population (Fig. S2 D). CEACAM-transfected cells supported strong attachment of Opa<sub>CEA</sub> *N. gonorrhoeae* (Fig. S2 E). In addition, CEACAM3 and CEACAM6 mediated internalization of Opa<sub>CEA</sub> gonococci within 30–60 min after infection (Fig. 2 A). In contrast, internalization mediated by CEACAM1 was <15% compared with CEACAM3, and more than 100-fold lower numbers of bacteria were recovered from control-transfected cells (pcDNA) (Fig. 2 A). Importantly, the internalization process via CEACAM3 and CEACAM6 depended on the correct phenotype of the bacteria, since nonopaque, piliated gonococci were barely taken up by these receptors (Fig. 2 B). These results indicated that both CEACAM3 and CEACAM6, but not CEACAM1, are able to mediate efficient internalization of Opa<sub>CEA</sub> gonococci when expressed in epithelial cells.

**CEACAM3, but not CEACAM6, Is Connected to the Small GTPase Rac.** The opsonin-independent phagocytosis of Opa<sub>CEA</sub>-expressing gonococci by human granulocytes is accompanied by cytoskeletal rearrangements leading to lamellipodia formation and the production of reactive oxygen metabolites (4, 11, 16), processes that are regulated by the small GTPase Rac (26). To analyze if CEACAM3- or CEACAM6-mediated phagocytosis might be connected to small GTPases of the Rho family, 293 cells were cotransfected with CEACAMs and with constructs encoding dominant-negative versions of Cdc42, Rac, and RhoA. Importantly, a strong decrease in CEACAM3-mediated uptake of opaque gonococci was observed upon coexpression of dominant-negative Rac (Rac N17; Fig. 3 A). In contrast, bacterial internalization via CEACAM6 was



**Figure 2.** Transient expression of CEACAMs in 293 cells allows interaction with Opa<sub>CEA</sub> gonococci. (A) 293 cells were transfected with CEACAM1, CEACAM3, CEACAM6, or the empty expression vector (pcDNA). After 2 d, cells were infected with Opa<sub>CEA</sub> gonococci. At the indicated time points, the number of internalized bacteria was determined by gentamicin protection assays. The graph shows mean values ± SDs of three independent experiments done in triplicate. (B) Transfected 293 cells were infected with nonopaque (Opa<sup>-</sup>), nonopaque, piliated (Opa<sup>-</sup>/P<sup>+</sup>), or Opa<sub>CEA</sub>-expressing (Opa<sub>CEA</sub>) gonococci for 1 h and analyzed in gentamicin protection assays. The graph shows mean values ± SDs of two independent experiments done in triplicate.



**Figure 3.** CEACAM3- but not CEACAM6-mediated internalization of gonococci depends on the small GTPase Rac. (A) 293 cells cotransfected with Cdc42 N17 or Rac N17 and either CEACAM3 or CEACAM6 were infected with Opa<sub>CEA</sub> gonococci, and the number of internalized bacteria was determined in gentamicin protection assays. The graph shows mean values  $\pm$  SDs of three independent experiments done in triplicate. Western blotting of whole cell lysates (WCL) with anti-myc tag antibody demonstrates expression of transfected Cdc42 N17 and Rac N17, respectively. (B) 293 cells, transfected with CEACAM3 or CEACAM6, were infected with Opa<sub>CEA</sub> *N. gonorrhoeae* for 1 h, fixed, and stained with polyclonal antibodies against gonococci and monoclonal antibodies against Rac. In CEACAM3-transfected cells, Rac is recruited to the site of bacteria-cell interaction (arrowhead).

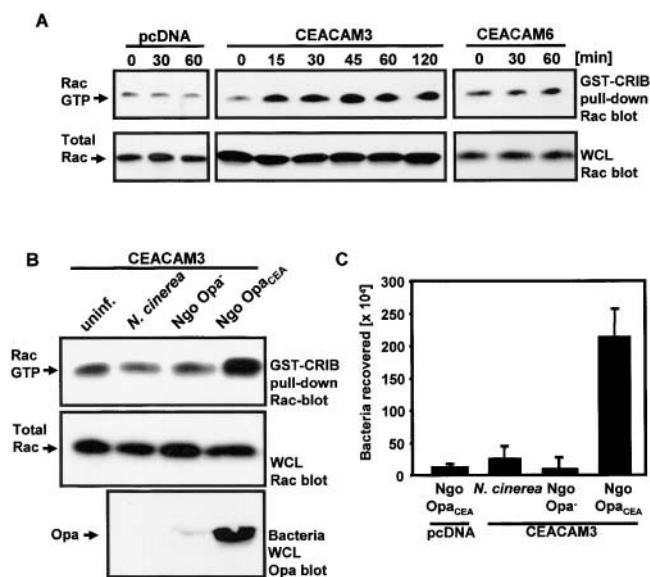
only marginally diminished by Rac N17, suggesting that CEACAM3 might be the granulocyte receptor that is connected to the small GTPase Rac. Interestingly, neither CEACAM3- nor CEACAM6-mediated internalization was compromised by coexpression of dominant-negative versions of the closely related small GTPases Cdc42hs (Fig. 3 A) or RhoA (unpublished data).

To investigate further if CEACAM3 is connected to Rac after *N. gonorrhoeae* infection, cells infected for 30 min were stained for gonococci and Rac. Importantly, cells transfected with CEACAM3 showed recruitment of Rac to the vicinity of attached Opa<sub>CEA</sub> bacteria (Fig. 3 B). In contrast, the distribution of Rac did not change in cells transfected with CEACAM6, despite the attachment of multiple gonococci (Fig. 3 B).

Biochemically, Rac stimulation is reflected by its GTP-loading status. Upon infection with Opa<sub>CEA</sub> gonococci, we observed a strong increase in GTP-Rac in CEACAM3-expressing 293 cells compared with uninfected cells (Fig. 4 A, top). This increase was already detectable 15 min after infection and reached maximum levels between 30 and 60 min paralleling the kinetics of efficient, CEACAM3-mediated internalization. In contrast, no increase in GTP-Rac was observed in control vector-transfected or in CEACAM6-expressing cells after infection (Fig. 4 A, top). An increase in Rac-GTP-loading was only detected in response to Opa<sub>CEA</sub>-expressing bacteria but not upon infec-

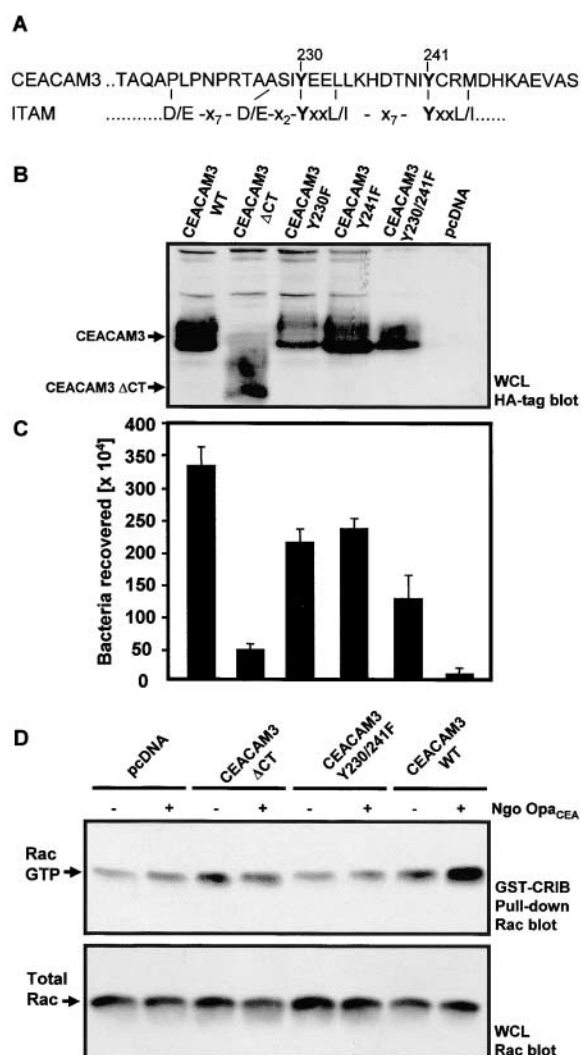
tion with nonopaque gonococci or nonpathogenic *N. meningitidis* (Fig. 4 B), demonstrating that it is the specific interaction between the bacterial Opa<sub>CEA</sub> adhesin and host CEACAM3 that mediates stimulation of Rac. Importantly, the levels of Rac-GTP induced by the different bacterial strains paralleled the internalization of these bacteria via CEACAM3 (Fig. 4 C). Together, these data corroborate the view that CEACAMs might be differentially connected to intracellular signaling pathways and that in particular CEACAM3 might be responsible for the stimulation of the small GTPase Rac in response to Opa<sub>CEA</sub> gonococci.

*The ITAM-like Sequence in the Cytoplasmic Domain of CEACAM3 Is Required for Opa<sub>CEA</sub>-mediated Uptake and Rac Stimulation.* Since the possession of the cytoplasmic domain is a distinctive feature of CEACAM3 that is absent from GPI-anchored CEACAM6, we wondered whether residues in the cytoplasmic domain of CEACAM3 are responsible for the internalization and/or for the observed stimulation of Rac. Therefore, a panel of CEACAM3 mutants were generated containing a deletion of the cytoplasmic domain ( $\Delta$ CT) or point mutations disrupting the ITAM-like sequence (Fig. 5 A) by substitutions of tyrosine for phenylalanine at either one or both positions (Y230F, Y241F, Y230/241F). In addition, a COOH-terminal HA tag was introduced. When 293 cells were transiently transfected with the respective constructs, equivalent levels of the proteins were expressed (Fig. 5 B). Importantly, uptake



**Figure 4.** CEACAM3 engagement by Opa<sub>CEA</sub> gonococci results in Rac stimulation. (A) 293 cells transfected with the indicated constructs were infected with Opa<sub>CEA</sub> *N. gonorrhoeae* for different time points. GTP-bound Rac was isolated using the Rac-binding domain of PAK (GST-CRIB) and detected by Western blotting with anti-Rac antibodies (top). Whole cell lysates (WCL) were also probed with anti-Rac antibodies (bottom). (B) CEACAM3-expressing 293 cells were left uninfected or were infected with *N. cinerea*, piliated, nonopaque *N. gonorrhoeae* (Ngo Opa<sup>-</sup>) or Opa<sub>CEA</sub> *N. gonorrhoeae* (Ngo Opa<sub>CEA</sub>). GST-CRIB pull-downs (top) or whole cell lysates (middle) were probed with anti-Rac antibodies. Lysates of the bacteria were probed with monoclonal anti-Opa antibody (bottom). (C) 293 cells transfected with the empty control vector (pcDNA) or CEACAM3 were infected with the bacteria employed in B and analyzed in gentamicin protection assays. The graph shows mean values  $\pm$  SDs of three independent experiments done in triplicate.

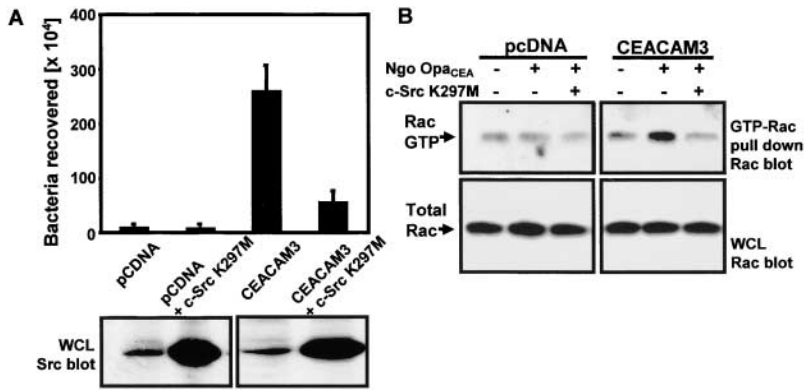
mediated by HA-tagged WT CEACAM3 was comparable to the internalization observed before with the untagged receptor, demonstrating that the addition of the HA tag did not alter the functionality of the protein (Fig. 5 C). However, deletion of the cytoplasmic domain of CEACAM3 ( $\Delta$ CT) led to almost complete abolishment of internalization of Opa<sub>CEA</sub> gonococci (Fig. 5 C). The ITAM-like sequence of CEACAM3 seemed to play a critical role, since substitution of either Y230 or Y241 resulted in  $\sim$ 30% reduction in bacterial uptake. Simultaneous mutation of both tyrosine residues resulted in a  $>$ 60% reduction of internalization (Fig. 5 C). Nevertheless, uptake mediated by CEACAM3 Y230/241F was consistently higher than internalization via CEACAM3  $\Delta$ CT, suggesting that additional residues within the cytoplasmic domain aside from the ITAM-like sequence can contribute to a low level of uptake. To analyze if the cytoplasmic domain is required for stimulation of Rac, pull-down analyses were performed. As observed before, infection of CEACAM3-expressing 293 cells with opaque gonococci resulted in enhanced GTP-loading of Rac (Fig. 5 D). In contrast, pcDNA, CEACAM3  $\Delta$ CT, or CEACAM3 Y230/241F-transfected cells did not respond to the bacterial challenge (Fig. 5 D) supporting the view that the ITAM-like se-



**Figure 5.** The ITAM-like sequence of CEACAM3 is critical for bacterial internalization and Rac stimulation. (A) Primary sequence of the COOH-terminal cytoplasmic domain of CEACAM3 containing the ITAM-like sequence. The ITAM consensus (31) is shown below with the critical tyrosine residues in bold. (B) 293 cells were transfected with the control vector (pcDNA) or the indicated HA-tagged CEACAM3 constructs. Whole cell lysates (WCL) were analyzed with anti-HA tag antibody. (C) Transfected cells as in B were infected with Opa<sub>CEA</sub>-expressing gonococci for 60 min and analyzed in gentamicin protection assays. The graph shows mean values  $\pm$  SDs of three to four independent experiments done in triplicate. (D) Cells were transfected as in B, and samples were lysed before or after 60 min of infection with Opa<sub>CEA</sub> *N. gonorrhoeae*. GST-CRIB pull-down assays (top) or whole cell lysates (WCL; bottom) of the samples were analyzed with monoclonal anti-Rac antibody.

quence in the cytoplasmic domain of CEACAM3 links receptor engagement with enhanced GTP-loading of Rac.

*Src Kinase Activity Is Critical for CEACAM3-mediated Uptake and Rac Stimulation.* Purified c-Src phosphorylates the CEACAM3 cytoplasmic domain in vitro (27), and Src PTKs are activated and phosphorylate CEACAM3 in granulocytes in response to opaque gonococci (17, 28). To analyze if Src PTKs play a role in CEACAM3-mediated bacterial internalization and Rac stimulation, a kinase-inactive



**Figure 6.** Src kinases connect CEACAM3 engagement with Rac stimulation. (A) 293 cells were transfected with the control vector (pcDNA), CEACAM3, or cotransfected with a kinase-inactive mutant of c-Src (c-Src K297M), infected with Opa<sub>CEA</sub> gonococci, and employed in gentamicin protection assays. The graph shows mean values  $\pm$  SDs of two independent experiments done in triplicate. Whole cell lysates (WCL) of the samples were analyzed for Src expression (bottom). (B) Lysates of cells as in A were prepared before and after infection with Opa<sub>CEA</sub> gonococci. GST-CRIB pull-down assays (top) or whole cell lysates (WCL; bottom) of the samples were analyzed with monoclonal anti-Rac antibody.

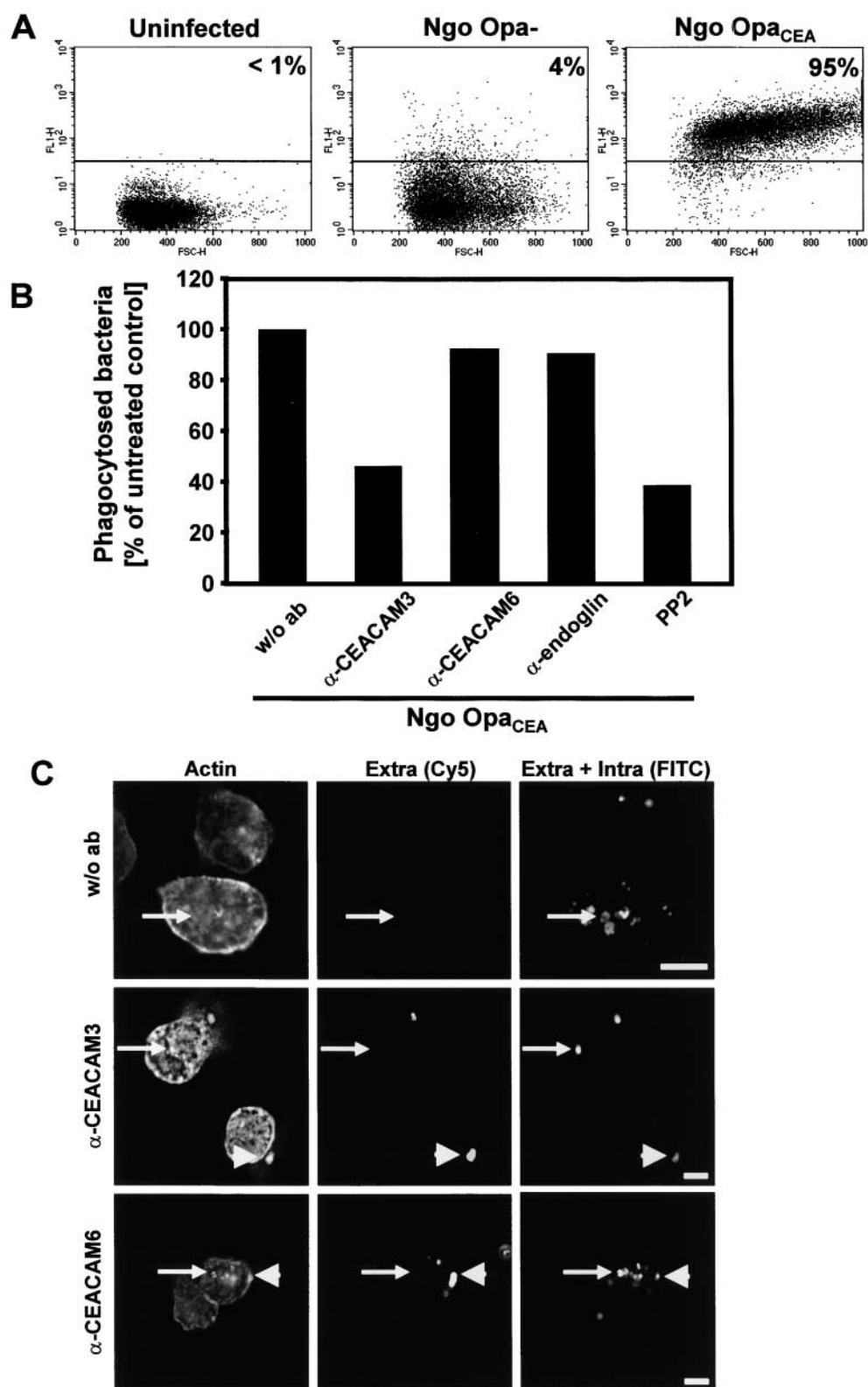
version of c-Src (Src K297M) was overexpressed in 293 cells. Notably, cells coexpressing CEACAM3 and Src K297M showed a prominent decrease in uptake of Opa<sub>CEA</sub> *N. gonorrhoeae* (Fig. 6 A). To investigate if Src PTK activity is upstream of Rac stimulation by CEACAM3, lysates were analyzed by pull-down assays with the GST-CRIB domain. Again, CEACAM3-transfected cells showed an increase in GTP-Rac upon infection with Opa<sub>CEA</sub> pathogens (Fig. 6 B). However, in cells coexpressing CEACAM3 and Src K297M the increase in GTP-Rac was completely suppressed (Fig. 6 B). Together, these results suggest that CEACAM3-mediated stimulation of the small GTPase Rac depends on Src kinase-initiated signaling via the CEACAM3 cytoplasmic domain ITAM-like sequence.

**Inhibition of CEACAM3 Blocks Efficient Phagocytosis of Opa<sub>CEA</sub> Gonococci by Human Granulocytes.** To address the functional significance of CEACAM3-initiated processes in human granulocytes, we analyzed the opsonin-independent phagocytosis of bacteria by primary granulocytes. Therefore, phagocytes were incubated with FITC-labeled bacteria, and internalization of bacteria was determined by FACS<sup>®</sup> analysis after quenching with trypan blue (23). Control experiments showed that addition of trypan blue reduced the fluorescence of extracellular bacteria by >90% (Fig. S1 A). As expected, nonopaque gonococci were only poorly phagocytosed (depending on the donor, 5–10% of the granulocyte population were FITC positive) and yielded a signal of intracellular fluorescence comparable to uninfected cells, whereas granulocytes readily internalized Opa<sub>CEA</sub>-expressing bacteria (75–95% of the granulocyte population) (Fig. 7 A). However, in the presence of the CEACAM3-specific monoclonal antibody a decreased phagocytosis of Opa<sub>CEA</sub> gonococci was observed, whereas addition of an isotype-matched control antibody against endoglin or addition of a CEACAM6-recognizing antibody only marginally interfered with internalization (Fig. 7 B). PP2, a specific pharmacological inhibitor of Src family PTKs, blocked the uptake of Opa<sub>CEA</sub> gonococci to the same extent as CEACAM3-specific antibodies (Fig. 7 B). This observation is in agreement with the view that CEACAM3-mediated phagocytosis requires Src PTK activity. The reduced fluorescence signal from intracellular bacteria in the presence of the CEACAM3-specific anti-

bodies was indeed due to decreased internalization of bacteria, as shown by differential staining of intra- and extracellular bacteria in infected samples (Fig. 7 C). In the absence of antibodies or in the presence of a CEACAM6-specific antibody, numerous Opa<sub>CEA</sub>-expressing *N. gonorrhoeae* were located inside the primary granulocytes. However, a marked reduction of intracellular bacteria could be observed in the presence of the CEACAM3-specific antibody (Fig. 7 C), suggesting that CEACAM3 is the predominant CEACAM family member responsible for the opsonin-independent uptake of CEACAM-binding gonococci by human granulocytes.

**Interference with CEACAM3 or Rac Stimulation Decreases Phagocytosis and Killing of Opa<sub>CEA</sub>-expressing Gonococci by Granulocytes.** If CEACAM3 is the major CEACAM family member contributing to the uptake of Opa<sub>CEA</sub> gonococci, then interference with Rac stimulation should also affect the internalization of the bacteria. Therefore, granulocytes were pretreated with a purified TAT fusion of dominant-negative Rac (TAT-RacN17),  $\beta$ -galactosidase (TAT-LacZ), or dominant-negative Cdc42 (TAT-Cdc42N17) (Fig. S1 B) and then incubated with FITC-labeled gonococci. Importantly, TAT-RacN17 severely diminished the opsonin-independent phagocytosis of Opa<sub>CEA</sub> *N. gonorrhoeae* by human granulocytes in a dose-dependent manner, whereas neither TAT-LacZ nor TAT-Cdc42N17 interfered with this process (Fig. 8 A). These results further support the hypothesis that CEACAM3 is the major granulocyte receptor of the CEACAM family mediating uptake of Opa<sub>CEA</sub> gonococci.

To investigate if the efficient CEACAM3-mediated phagocytosis finally leads to the killing and elimination of the microorganisms, primary human granulocytes were incubated with Opa<sub>CEA</sub> gonococci, and the amount of viable bacteria was determined after 60 min of coincubation. As observed before, ~60% of the inoculated Opa<sub>CEA</sub>-expressing, but not nonopaque gonococci, were eliminated by the phagocytes within 60 min (Fig. 8 B). However, when the CEACAM3-specific antibody was added before infection an improved survival of Opa<sub>CEA</sub> gonococci could be observed, whereas addition of the isotype-matched control antibody or a CEACAM6-recognizing antibody only marginally altered the survival of the bacteria (Fig. 8 B). To-

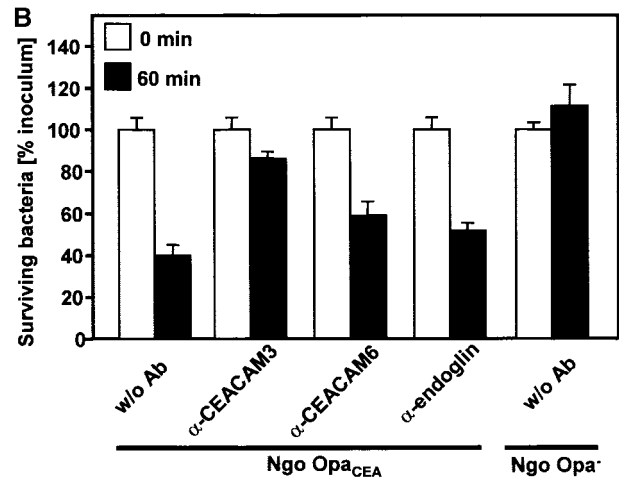
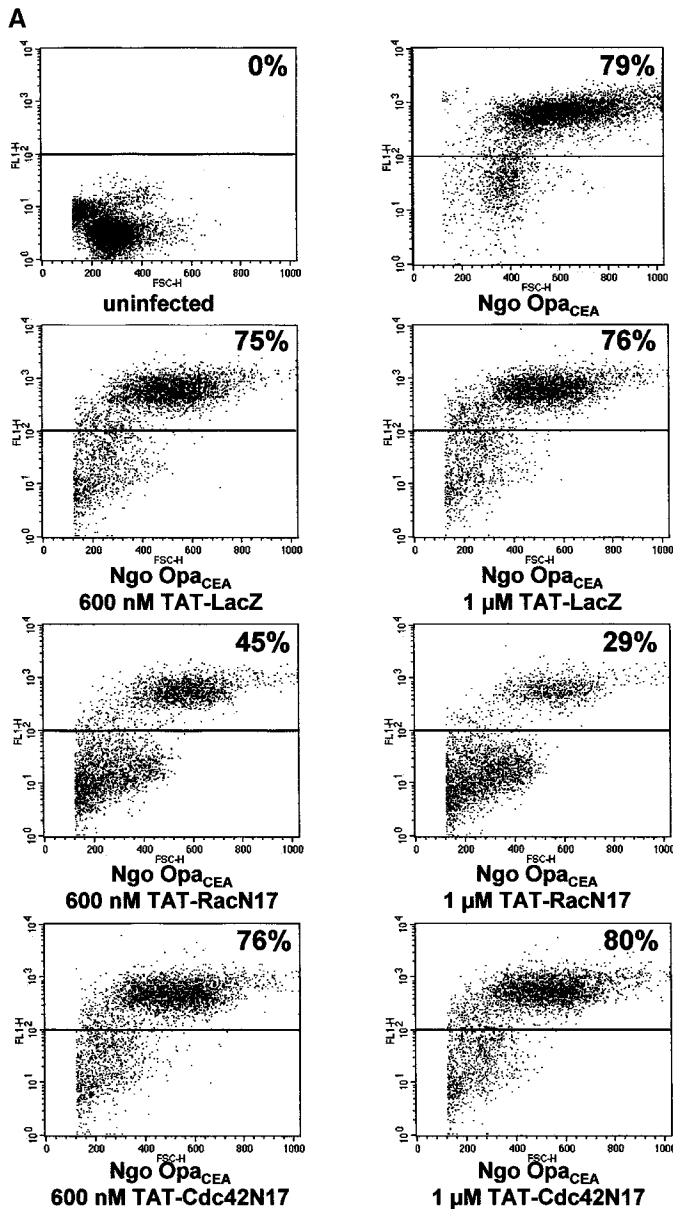


**Figure 7.** CEACAM3 is critical for uptake of CEACAM-binding bacteria by primary human granulocytes. (A) Granulocytes were incubated with FITC-labeled Opa<sub>CEA</sub> gonococci (Ngo Opa<sub>CEA</sub>) or nonopaque, piliated gonococci (Ngo Opa<sup>-</sup>), respectively, or left uninfected. After 20 min, phagocytosis was measured by FACS<sup>®</sup> analysis. Values represent the percentage of granulocytes containing FITC-labeled bacteria from a representative experiment. Similar values were obtained with granulocytes from three different donors. (B) Phagocytosis of Opa<sub>CEA</sub> bacteria was analyzed as above in the absence or presence of 20  $\mu$ g of the indicated monoclonal antibodies or 10  $\mu$ M of the Src PTK inhibitor PP2. The graph shows mean values of a representative experiment done in triplicate. Equivalent results were obtained with granulocytes isolated from three different donors. (C) Human granulocytes were infected with FITC-labeled Opa<sub>CEA</sub> gonococci in the presence or absence of 20  $\mu$ g of the indicated monoclonal antibodies for 60 min. Cells were differentially stained for extracellular (arrowheads) and intracellular bacteria (small arrow). Cellular actin was visualized with phalloidin-TRITC.

gether, these results suggest that CEACAM3-mediated Rac stimulation is a major pathway leading to the opsonin-independent uptake and elimination of CEACAM-binding gonococci by human granulocytes.

*CEACAM3 Mediates Rac Stimulation and Phagocytosis in Response to H. influenzae and M. catarrhalis.* To address if CEACAM3 plays a significant role in the elimination and control of other CEACAM-binding pathogens, we inves-





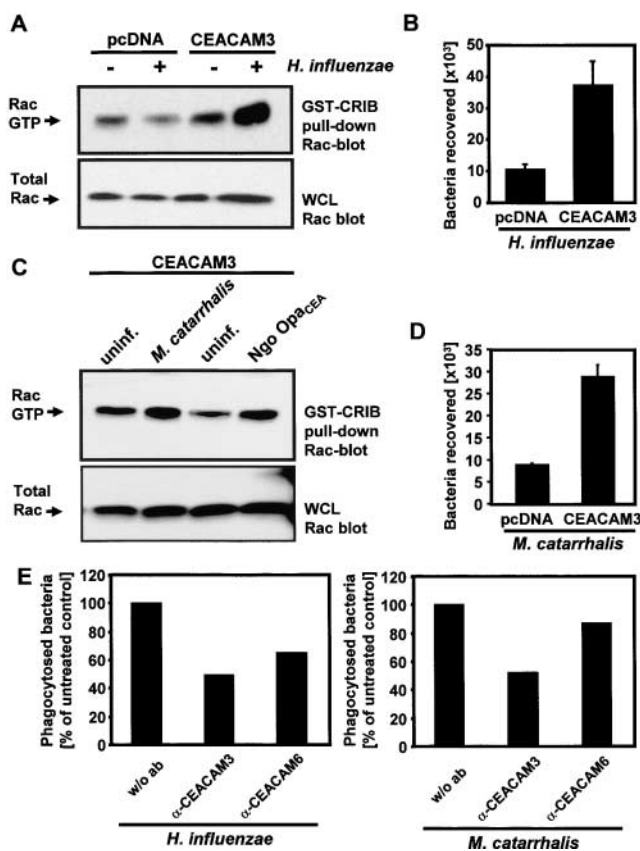
**Figure 8.** CEACAM3 and Rac stimulation are required for efficient uptake and elimination of  $Opa_{CEA}$  gonococci by primary human granulocytes. (A) Granulocytes were either untreated or pretreated with the indicated amounts of TAT-LacZ, TAT-RacN17, or TAT-Cdc42N17 and infected with FITC-labeled  $Opa_{CEA}$  gonococci (Ngo  $Opa_{CEA}$ ). Phagocytosis was determined by FACS<sup>®</sup> analysis, and the graphs show the result of a representative experiment. Similar results were obtained with granulocytes isolated from three different donors. (B) Granulocytes were incubated with  $Opa_{CEA}$  gonococci (Ngo  $Opa_{CEA}$ ) or nonopaque, piliated gonococci (Ngo  $Opa^{-}$ ), respectively, in the absence or presence of 20  $\mu$ g of the indicated antibodies. Before and after 60 min of incubation, aliquots of the samples were plated on GC agar to determine the number of viable gonococci. The graph shows mean values  $\pm$  SDs of a representative experiment done in triplicate. Equivalent results were obtained with granulocytes isolated from three different donors.

tigated if *M. catarrhalis* or *H. influenzae* are recognized by CEACAM3 and if they trigger Rac-GTP loading. Indeed, in CEACAM3-expressing 293 cells a nonencapsulated variant of *H. influenzae* strain RD stimulated a strong increase in Rac-GTP upon infection, whereas no such increase was observed in cells transfected with the empty control vector (Fig. 9 A). Again, Rac-GTP loading in response to *H. influenzae* was correlated with enhanced CEACAM3-mediated internalization of these pathogens, albeit at lower levels than observed in response to  $Opa_{CEA}$  gonococci (Fig. 9 B). Moreover, a UspA1-positive *M. catarrhalis* strain enhanced Rac-GTP loading and was internalized by 293 cells in a CEACAM3-dependent manner (Fig. 9, C and D). Interference with CEACAM3, and to a lesser extent with CEACAM6, on primary human granulocytes decreased phagocytosis of *H. influenzae*, and uptake

of *M. catarrhalis* was strongly diminished by CEACAM3-specific antibodies (Fig. 9 E), suggesting that CEACAM3 is involved in clearance of diverse microorganisms that target human CEACAMs. Together, these data support the view that CEACAM3 is a major granulocyte receptor responsible for Rac stimulation by an opsonin-independent phagocytosis of diverse CEACAM-binding, human-specific pathogens.

## Discussion

The human pathogens *N. gonorrhoeae*, *N. meningitidis*, *M. catarrhalis*, and *H. influenzae* avoid acquired immune defenses of their host by sophisticated mechanisms such as variation of surface structures or secretion of IgA-specific proteases. However, despite widespread colonization with some of these



**Figure 9.** *M. catarrhalis* and *H. influenzae* are recognized and phagocytosed via CEACAM3. (A) Empty control vector (pcDNA) or CEACAM3-transfected 293 cells were left uninfected or were infected with unencapsulated *H. influenzae* strain RD for 60 min. GST-CRIB pull-downs (top) or whole cell lysates (bottom) were probed with anti-Rac antibodies. (B) Cells as in A were infected with *H. influenzae* strain RD and analyzed in gentamicin protection assays. The graph shows mean values  $\pm$  SDs of two independent experiments done in triplicate. (C) CEACAM3-transfected 293 cells were left uninfected or were infected with *M. catarrhalis* strain 11994 or with Opa<sub>CEA</sub> gonococci (Ngo Opa<sub>CEA</sub>) for 60 min. GST-CRIB pull-downs (top) or whole cell lysates (bottom) were probed with anti-Rac antibodies. (D) 293 cells transfected with the empty control vector (pcDNA) or CEACAM3 were infected with *M. catarrhalis* for 60 min and analyzed in gentamicin protection assays. The graph shows mean values  $\pm$  SDs of two independent experiments done in triplicate. (E) Human granulocytes were infected with FITC-labeled *M. catarrhalis* or *H. influenzae*, respectively, in the presence or absence of the indicated antibodies for 20 min and analyzed by FACS<sup>®</sup> for intracellular bacteria. The graphs show the results of representative experiments. Similar results were obtained with granulocytes isolated from two different donors.

microorganisms, severe disease outcomes occur only in a small portion of the host population, suggesting that innate immune defenses are able to contain the bacteria in most cases. We demonstrate here that human granulocytes not only recognize CEACAM-binding variants of these pathogens but that bacterial engagement of a granulocyte-specific member of the CEACAM family, CEACAM3, initiates phagocyte effector mechanisms leading to the elimination of the microbes.

It has been observed previously that human granulocytes can recognize opaque variants of pathogenic *Neisseriae* in an opsonin-independent manner (29) and that this recognition depends on members of the CEACAM family (4, 5, 17).

Overexpression of individual CEACAM family members in nonphagocytic cells has revealed that several CEACAMs can mediate uptake in such a cellular background (11, 12, 18). However, the role of individual receptors in the interaction of granulocytes with CEACAM-binding pathogens has not been analyzed. The data presented in this study provide for the first time biochemical and functional evidence that CEACAM3 is the predominant CEACAM family member on human granulocytes mediating elimination of CEACAM-binding bacteria. Since there is no endogenous ligand known for CEACAM3 (13), the recognition of pathogenic *Haemophilus*, *Moraxella*, and *Neisseria* species now assigns a function to this orphan receptor of the innate immune system.

The efficient CEACAM3-mediated clearance of bacterial pathogens seems to be closely linked to the ability of this receptor to stimulate the small GTPase Rac, a critical regulator of actin polymerization, phagocytosis, and the oxidative response of granulocytes (30). Though CEACAM6 can mediate internalization when overexpressed in epithelial cell lines (11, 12), bacterial engagement of CEACAM6 did not lead to Rac stimulation, and interference with CEACAM6 did not block the clearance of CEACAM-binding bacteria by human granulocytes. The difference between CEACAM3- and CEACAM6-mediated events is due to the presence of an ITAM-like sequence in the CEACAM3 cytoplasmic tail. ITAMs have been characterized in several receptors belonging to the Ig superfamily (31). ITAM function depends on the phosphorylation of key tyrosine residues that is often mediated by Src family protein tyrosine kinases (32). Interestingly, tyrosine residue Y241 within the CEACAM3 ITAM-like sequence is embedded in the context of YxxM, where a methionine residue is located at the +3 position with respect to the tyrosine (compared with YxxL/I in the canonical ITAM [31]). Such a YxxM sequence has been shown to serve as a preferred docking site for SH2 domains of the p85 subunit of phosphatidylinositol 3'-kinase (PI-3K) (33). Therefore, we would predict that phosphorylated Y241 connects phosphorylation of CEACAM3 to the reported recruitment of PI-3K (34). Since the products of PI-3K can activate several Rac GEFs, such as Vav and SWAP70 (35, 36), PI-3K binding is a potential molecular link between CEACAM3 ITAM phosphorylation and downstream Rac stimulation.

CEACAM3 does not engage in homo- or heterotypic interactions with other CEACAM family members, and endogenous ligands are unknown (37). In light of the results presented in this paper, CEACAM3 is best viewed as a specifically adapted single chain phagocytic receptor involved in the clearance of CEACAM-binding bacteria by human granulocytes. Most of the known CEACAM-binding bacterial species are human-specific pathogens. Likewise, the expression of CEACAM3 is also restricted to human granulocytes, and no homologues of CEACAM3 have been detected in rodents or nonhuman primates (38, 39), suggesting a striking coevolution of human innate immune defenses with a restricted set of specialized pathogens. It is tempting to speculate that gonorrhea-associated complica-

tions effecting reproduction such as early trimester abortion or infertility (40) might have provided a selective advantage to individuals able to counteract CEACAM-binding bacteria by innate immune defenses. On the other hand, pathogens might evolve variants of their CEACAM-binding adhesins that retain association with CEACAM family members expressed on epithelial cells (such as CEA or CEACAM1) but that loose recognition by CEACAM3. Such adhesins would allow attachment to and invasion of epithelial cells but would avoid elimination by granulocytes. Indeed, several meningococcal and gonococcal Opa<sub>CEA</sub> proteins have been characterized that exhibit this property (11, 12, 41, 42). It will be interesting to investigate whether the ability to cause severe forms of disease is correlated with the expression of CEACAM-binding adhesins that are not recognized by CEACAM3.

Together, the results presented in this paper describe the orphan receptor CEACAM3 as a novel single-chain phagocytic receptor of the innate immune system. Since internalization via CEACAM3 is opsonin independent, this receptor should be of particular importance in the control of bacteria that do not induce protective acquired immune responses. Since multiple commensal and pathogenic bacteria found in association with human mucosal surfaces are exploiting CEACAM family members (43–45), CEACAM3-mediated, opsonin-independent phagocytosis by granulocytes might be a general defense mechanism evolved by the human innate immune system directed against CEACAM-binding microorganisms.

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