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The Granulocyte Receptor Carcinoembryonic Antigen-Related Cell Adhesion Molecule 3 (CEACAM3) Directly Associates with Vav to Promote Phagocytosis of Human Pathogens[1]

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The human granulocyte-specific receptor carcinoembryonic antigen-related cell adhesion molecule (CEACAM)3 is critically involved in the opsonin-independent recognition of several bacterial pathogens. CEACAM3-mediated phagocytosis depends on the integrity of an ITAM-like sequence within the cytoplasmic domain of CEACAM3 and is characterized by rapid stimulation of the GTPase Rac. By performing a functional screen with CEACAM3-expressing cells, we found that overexpression of a dominant-negative form of the guanine nucleotide exchange factor Vav, but not the dominant-negative versions SWAP70, Dock2, or ELMO1 interfered with CEACAM3-initiated phagocytosis. Moreover, small interfering RNA-mediated silencing of Vav reduced uptake and abrogated the stimulation of Rac in response to bacterial CEACAM3 engagement. In Vav1/Vav2-deficient cells, CEACAM3-mediated internalization was only observed after re-expression of Vav. Vav colocalized with CEACAM3 upon bacterial infection, coimmunoprecipitated in a complex with CEACAM3, and the Vav Src homology 2 domain directly associated with phosphorylated Tyr^{230} of CEACAM3. In primary human granulocytes, TAT-mediated transduction of dominant-negative Vav, but not SWAP70, severely impaired the uptake of CEACAM3-binding bacteria. These data support the view that, different from canonical ITAM signaling, the CEACAM3 ITAM-like sequence short-wires bacterial recognition and Rac stimulation via a direct association with Vav to promote rapid phagocytosis and elimination of CEACAM-binding human pathogens. ***The Journal of Immunology,*** **2007, 178: 3797–3805.**

Bacterial pathogens *Neisseria gonorrhoeae* and *Neisseria meningitidis* belong to the few Gram-negative pathogens that are exquisitely adapted to humans as their sole natural host. Both are highly efficient colonizers of the human mucosa, which they use as a platform for transmission to the next person (1, 2). A number of well-characterized adhesive factors allow gonococci and meningococci to specifically contact human cells and to establish a dependable foothold on the epithelium (3). Conversely, type IV pili mediate the initial long-range contact with the mucosal surface. In a second step, the so-called colony opacity-associated (Opa)[4] proteins provide a more intimate adhesion. Work over the last decade has identified the cellular targets of neisserial Opa proteins. Although some Opa proteins bind to heparan sulfate proteoglycans, the majority of Opa proteins (Opa_{CEA}) recognize one or several members of the human family of carcinoembryonic antigen-related cell adhesion molecules (CEACAMs) (reviewed in Ref. 4). Furthermore, other human-specific Gram-negative pathogens that colonize mucosal surfaces, such as *Haemophilus influenzae* or *Moraxella catarrhalis*, also express CEACAM-binding adhesins (5–7). In addition to host cell adhesion, bacterial engagement of CEACAMs triggers specific gene expression events in epithelial cells, which appear to facilitate mucosal colonization (8). Furthermore, pathogen-mediated stimulation of CEACAM1 expressed by different hemopoietic cell types interferes with acquired immune defense mechanisms by triggering inhibitory signaling (reviewed in Ref. 9).

In sharp contrast to the potential benefits neisseriae might gain from CEACAM recognition, a series of studies has demonstrated that CEACAM-binding bacteria are effectively phagocytosed in an opsonin-independent manner by human granulocytes (10–13). These cells represent the first line of defense against neisseriae, and the purulent inflammation is a diagnostic hallmark of both gonorrhea and bacterial meningitis (1, 2). Interestingly, the opsonin-independent recognition of gonococci by granulocytes does also depend on CEACAMs (11, 12, 14). In particular, a granulocyte-specific member of the CEACAM family, CEACAM3, was identified as the major receptor responsible for opsonin-independent phagocytosis and bacterial elimination (15). Studies on the structure-function relationship of CEACAM3 point to an ITAM-like sequence within the cytoplasmic domain that is phosphorylated upon receptor engagement (15, 16). Tyrosine phosphorylation of the CEACAM3 ITAM-like sequence is mediated by active protein tyrosine kinases (PTKs) of the Src family, in particular Hck and Fgr, that have been found to be strongly activated in primary human granulocytes in response to CEACAM-binding bacteria

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[4] Abbreviations used in this paper: Opa, opacity-associated; dn, dominant negative; GEF, guanine nucleotide exchange factor; PTK, protein tyrosine kinase; RFP, red fluorescent protein; HA, hemagglutinin; SH2, Src homology 2; CEACAM, carcinoembryonic antigen-related cell adhesion molecule; siRNA, small interfering RNA; WT, wild type.

(12). In turn, the integrity and the tyrosine phosphorylation of the CEACAM3 ITAM-like sequence are critical for stimulation of the small GTPase Rac as well as for CEACAM3-mediated bacterial internalization (15, 16). However, the molecular link between CEACAM3 engagement, tyrosine phosphorylation, and increased GTP-loading of Rac is not known.

In this study we provide experimental evidence that the guanine nucleotide exchange factor (GEF) Vav links CEACAM3 engagement by CEACAM-binding *N. gonorrhoeae* with the stimulation of the small GTPase Rac. Importantly, Vav directly associates via its Src homology 2 (SH2) domain with a phosphorylated tyrosine residue within the ITAM-like sequence of the receptor. Interference with Vav reduces granulocyte uptake of CEACAM-binding bacteria suggesting that the short-wiring of CEACAM3 engagement with Vav recruitment and Rac stimulation is critical in the control of human-specific pathogens by the innate immune system.

Materials and Methods

Bacteria

Opa_{CEA}-expressing (Opa_{52}), nonpiliated *N. gonorrhoeae* MS11-B2.1 (strain N309) and nonopaque, piliated gonococci MS11 were provided by T. F. Meyer from Max-Planck-Institut (MPI) für Infektionsbiologie (Berlin, Germany). *Neisseria* were grown on GC agar (Invitrogen Life Technologies) supplemented with vitamins at 37°C, 5% CO_2 and subcultured daily. Opa protein expression was monitored by phenotypic examination of agar colonies as well as by Western blotting with monoclonal anti-Opa Ab (clone 4B12/C11) obtained from M. Achtman (MPI für Infektionsbiologie, Berlin, Germany). For phagocytosis assays, bacteria were labeled with 0.2 μg/ml CFSE (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_2. Cells were subcultured every 3–4 days. HeLa cells with stable expression of CEACAM3 (17) were obtained from W. Zimmermann (Universität München, München, Germany) and cultured in DMEM, 10% FCS, and 500 μg/ml G418. Mouse embryonic fibroblasts were isolated at day 12.5 postcoitum from $Vav1^{-/-}Vav2^{-/-}$ mouse embryos backcrossed to C57BL/6 mice for over 10 generations (18). Head and inner organs were removed and the remaining bodies were minced and treated with trypsin/EDTA solution at 37°C. The resulting primary fibroblasts were immortalized at passage 2 by retroviral transduction with SV40 large T Ag provided by C. Brakebusch (MPI Biochemie, Munchen, Germany). Mouse embryonic fibroblasts were grown on gelatin-coated culture dishes in DMEM containing 10% FCS, nonessential amino acids as well as sodium pyruvate and were used between passage 4 and passage 8. Before infection and Rac pull-down assays, all cells were serum-starved overnight in DMEM containing 0.5% calf serum. Primary human granulocytes were purified from freshly drawn blood as described previously (15). Viability of cells was determined before infection using trypan blue staining and in all cases was >90%.

Recombinant DNA constructs

Mammalian expression plasmids encoding v-Src (derived from the Prague C strain of Rous sarcoma virus) or hemagglutinin (HA)-tagged CEACAM3 and the derived HA-tagged CEACAM3 mutants have been described previously (15, 19). CEACAM3-red fluorescent protein (RFP) was constructed by amplifying CEACAM3 with primers CGM1-RFP (sense) 5′-ATAGCTAGCGCCACCATGGGGCCCCCCTGAGCCTCTCCCCAC-3′ and CGM1-RFP (antisense) 5′-ATAACCGGTGAAGCCACTTCTGCTTTGTGGTCCATCCG-3′ and subcloning via the *Nhe*I and the *Age*1 sites into pDs-Red (BD Clontech). CEACAM3-GFP was derived after PCR amplification of HA-tagged wild-type (WT) CEACAM3 (CEACAM3 WT) with primers CEACAM3-IF (sense) 5′-GAAGTTATCAGTCGATACCATGGGGCCCCCCTCAGCC-3′ and (antisense) 5′-ATGGTCTAGAAAGCTTGCAGCGTAATCTGGAACGTCATATGG-3′ followed by insertion into vector pDNR-dual using the In-Fusion Cloning kit (BD Clontech). CEACAM3 cDNA was then mobilized from pDNR-dual by Cre-mediated recombination into pLPS-3′ enhanced GFP (BD Clontech) to allow expression of GFP-tagged CEACAM3 in eukaryotic cells. Plasmids encoding HA-tagged Vav1 and Vav2 were provided by S. Moores (Harvard Medical School, Boston, MA) and plasmid encoding human Dock2 was from S. Tanaka (Hokkaido University, Sapporo, Japan). Plasmids containing full-length human cDNA of SLP76 (SH2-domain-containing leukocyte protein of 76 kDa; IRATp970H0521D), ELMO1 (DKFZp 434B0819), and SWAP70 (IMAGp958H17135q2), as well as the partial cDNA of Vav1 (IMAGp958K22536q2) were obtained from the Ressourcen Zentrum Primär Datenbank.

Dominant-negative (dn) constructs of Vav1, ELMO1, Dock2, and SWAP70 were generated by PCR with primers VAV-IF (sense) 5′-GAAGTTATCAGTCGACGGTACCTTCTATCAGGGCTACC-3′ and (antisense) 5′-ATGGTCTAGAAAGCTTTCAGCAGTATTCAGAATAATCTTCC-3′; ELMO-IF (sense) 5′-GAAGTTATCAGTCGACATGCCGCCACCCGCGGAC-3′ and (antisense) 5′-ATGGTCTAGAAAGCTTTCACATATGAGGGCAGTCCTTTC-3′; Dock2-IF (sense) 5′-GAAGTTATCAGTCGACATGGCCCCCTGGCGCAAAGC-3′ and (antisense) 5′-ATGGTCTAGAAAGCTTTCAGTCATGGAATCCATCGTGTAGAG-3′; and SWAP70-IF (sense) 5′-GAAGTTATCAGTCGACATGGGGAGCTTGAAGGAGGAGC-3′ and (antisense) 5′-ATGGTCTAGAAAGCTTCTACTGCAGCTTTAGGTACATGTCTTCC-3′, respectively. The resulting PCR fragments were inserted into vector pDNR-dual using the In-Fusion Cloning kit (BD Clontech). The cDNA was then mobilized from pDNR-dual by Cre-mediated recombination into pLP-CMV-*myc* (BD Clontech) to allow expression of *myc*-tagged proteins in eukaryotic cells.

GST-Vav1-dn was generated using oligonucleotides Vav1-dn-*Eco*R1-pGEX (sense) 5′-ATAGAATTCGGTACCTTCTATCAGGGCTACC-3′ and Vav1-dn-*Xho*I-pGEX (antisense) 5′-ATACTCGAGTCAGCAGTATTCAGAATAATCTTCC-3′ and subcloned into the *Eco*R1 and *Xho*I restriction sites of pGEX4-T1 (Amersham Biosciences). GST-Vav-SH2 was produced by PCR amplification of Vav1 with primers Vav1-SH2-IF (sense) 5′-GAAGTTATCAGTCGACCCTCAGGACCTGTCTGTTC-3′ and Vav1-SH2-IF (antisense) 5′-ATGGTCTAGAAAGCTTATCTCTTTTCAGGCTCCTTG-3′ and inserted into vector pDNR-dual using the InFusion Cloning kit (BD Clontech). The cDNA was then mobilized from pDNR-dual by Cre-mediated recombination into pGEX4-T1 loxP derived from pGEX4-T1 using the Creator Vector construction kit (BD Clontech). GST-SLP76-SH2 was produced accordingly using primers SLP76-SH2-IF (sense) 5′-GAAGTTATCAGTCGACCCCGCGGAGGAAGAG-3′ and (antisense) 5′-ATGGTCTAGAAAGCTTCTATGGGTACCCTGCAGCATG-3′. TAT-HA-Vav1-dn was generated by PCR using primers Vav1-dn-*Xho*I (sense) 5′-TTATCTCGAGGGTACCTTCTATCAGGGCTACC-3′ and (antisense) 5′-TTATGAATTCTCAGCAGTATTCAGAATAATCTTCC-3′ and subcloning into pTAT-HA provided by S. Dowdy (University of California, San Diego, La Jolla, CA). SWAP70-dn was cloned into pTAT-HA using *Nco*I and *Xho*I restriction sites after amplification with primers SWAP70-dn-*Nco*I (sense) 5′-TTATCCATGGGGAGCTTGAAGGAGGAGC-3′ and SWAP70-dn-*Xho*I (antisense) 5′-TTATCTCGAGTCACTGCAGCTTTAGGTACATGTCTTCC-3′.

Transfection of cells, cell lysis, Western blotting, and determination of Rac GTP-loading

Transfection of 293T cells, cell lysis, Western blotting, and determination of Rac GTP-loading were performed as described previously (15). Vav1/Vav2-deficient fibroblasts were transfected with LipofectaminePlus (Invitrogen Life Technologies) in 24-well plates according to the manufacturer's instructions using 1.5 μg of the empty control vector pCF1 or pCF1 Vav1-WT-HA and 0.5 μg of plasmid encoding CEACAM3-RFP. Transfected cells were used in infection experiments 48 h later. mAbs used in Western blotting experiments against Rac (clone 23A8), against phosphotyrosine (clone 4G10), and against v-Src (clone EC10) were from Upstate Biotechnology, mAb against β-actin (clone AC-74) was from Sigma-Aldrich, mAb against GFP (clone JL-8) was from BD Clontech, mAb against GST (clone B-14), mAb against Vav (clone B2), and rabbit polyclonal anti-Vav2 Abs (H-200) were from Santa Cruz Biotechnology. Monoclonal Ab against the HA-tag (clone 12CA5) and against the *myc*-epitope (clone 9E10) were purified from hybridoma supernatants. A rabbit polyclonal Ab generated against formaldehyde-fixed *N. gonorrhoeae* and *N. meningitidis* (clone IG511) was produced by immunoGlobe (Himmelstadt, Germany). Secondary Abs were from Jackson ImmunoResearch Laboratories.

Gentamicin protection assay

For gentamicin protection assays, 6×10^5 293 cells in 24-well plates were infected with 20 bacteria/cell for 60 min. Following 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.

RNA interference treatment

A small interfering RNA (siRNA)-oligonucleotide directed against Vav2 was obtained from Dharmacon, which targeted the sequence 5′-ggacat caacttccggccg-3′. A control siRNA-oligonucleotide was obtained from T. F. Meyer (MPI Infektionsforschung, Berlin, Germany) and was designed against the sequence 5′-aacgtacgcggaatacttcgatt-3′ in firefly luciferase. HeLa cells in 24-well plates were treated with a mixture of 2 μl of Oligofectamin (Invitrogen Life Technologies) and 2.5 μl of a 20-μM stock-solution of the indicated siRNA in 250 μl of OptiMEM (Invitrogen Life Technologies). After 4 h, 125 μl of OptiMEM with 30% FCS were added and the cells incubated for another 24 h before the medium was replaced with regular growth medium. Two days after the start of the treatment, cells were lysed for Western blot analysis or used in infection experiments.

Immunofluorescence staining and microscopy

Immunofluorescence labeling was performed as described previously (15). For differentiating between extracellular and intracellular bacteria, cells were infected with fluorescein-labeled gonococci and fixed samples were stained before permeabilization with polyclonal Abs against gonococci (clone IG511) and Cy5-coupled goat anti-rabbit Abs, resulting in fluorescein-labeled intracellular and fluorescein/Cy5-labeled extracellular bacteria. In the case of Vav2-HA transfected 293 cells and TAT-protein transduced granulocytes, the samples were further stained after permeabilization with monoclonal anti-HA-tag Abs followed by Cy5- or Cy3-labeled goat anti-mouse Abs to detect the transfected or transduced cells, respectively. Samples were viewed using a LSM 510 laser scanning confocal microscope (Zeiss). For all triple-labeled specimens, the signals of the fluorescent dyes were serially recorded to avoid bleed-through due to simultaneous excitation. The corresponding images were digitally processed with Photoshop6 (Adobe Systems) and merged to yield pseudo-colored RGB pictures.

Immunoprecipitation and GST-pull-down

For immunoprecipitation, 3 μg of the indicated Abs were added to whole cell lysates and incubated for 4 h under rotation at 4°C. The 30 μl of protein A/G plus-Sepharose (Santa Cruz Biotechnology) were added to each sample for 1 h, before precipitates were washed three times with modified radioimmunoprecipitation assay buffer and mixed with 2× SDS-sample buffer (15). For GST-pull-downs, 10 μg of purified GST or GST-fusion protein attached to glutathione-Sepharose were added to whole cell lysates and incubated for 2 h at 4°C. After four washes with modified radioimmunoprecipitation assay buffer, precipitates were boiled in SDS sample buffer, before SDS-PAGE and Western blot analysis.

Generation and Far Western probing of peptide spot membranes

The 15-mer peptides surrounding Tyr^{230} or Tyr^{241} of the CEACAM3 cytoplasmic domain (see Fig. 6*B*) were assembled by SPOT synthesis on an AC-S01 cellulose membrane (AIMS Scientific Products) as described previously (20). Phosphotyrosine was incorporated as *N*-α-Fmoc-*O*-(bis(dimethylamino)phosphono)-L-tyrosine derivative, and protecting groups were removed by an additional treatment with 90% aqueous trifluoroacetic acid overnight. The peptides stay anchored via their C termini to the polyethyleneglycol spacer of the membrane and are N-terminally acetylated.

Peptide spot membranes were blocked with blocking buffer (1 volume Sigma-Aldrich casein-based blocking buffer, 4 volumes Tris-buffered saline (pH 8.0) containing 0.05% Tween (TBST), and 5% saccharose) for 16 h at 4°C and then incubated with 20 μg of GST-Vav-dn or GST in blocking buffer for 20 h. After several washes in TBST, GST was detected by anti-GST Ab, followed by HRP-coupled goat anti-mouse Ab and visualized by ECL.

Expression and purification of GST- and TAT-fusion proteins

GST-fusion as well as TAT-fusion constructs were purified from *Escherichia coli* BL-21 as previously described (15) using glutathione-agarose FF (Amersham Biosciences) for GST-containing proteins or Ni^{2+}NTA columns (Qiagen) for His-tagged, TAT-fusion proteins, respectively. After elution of TAT-fusion proteins with 8 M urea, 100 mM NaCl, 20 mM HEPES, and 500 mM imidazole, 10% glycerol was added. Samples were rapidly desalted and buffer was exchanged to RPMI 1640 containing 10% glycerol using a PD-10 column (Amersham Biosciences). Purity of the preparations was examined by SDS-PAGE and the protein concentration estimated based on a BSA standard.

Granulocyte phagocytosis

Phagocytosis was determined by flow cytometry as described previously (15). Briefly, granulocytes were incubated for 1 h at 37°C with the indicated amounts of purified TAT-fusion proteins before 1×10^6 granulocytes were infected with 2×10^7 fluorescein-labeled bacteria in 1 ml of phagocytosis buffer (1× PBS, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5 mM glucose, 1% heat-inactivated serum) for 15 min at 37°C. Phagocytosis was stopped by addition of ice-cold phagocytosis buffer, samples were washed, taken up in ice-cold PBS, 2% FCS, 2 mg/ml trypan blue and analyzed on a FACSCalibur (BD Biosciences). To obtain an estimate of the amount of phagocytosed bacteria (uptake index), the percentage of fluorescein-positive granulocytes was multiplied by the mean fluorescence of these cells. For microscopic analysis, infected granulocytes were fixed with 4% paraformaldehyde in PBS and stained as described.

Results

CEACAM3-mediated phagocytosis is compromised by Vav-dn

To get insight into the molecular connection between CEACAM3 engagement and Rac stimulation, we sought to identify the GEF responsible for increased Rac GTP loading in response to bacterial contact. Therefore, we coexpressed CEACAM3 with dominant-interfering mutants of the Rac GEFs Vav, SWAP70, ELMO1, and Dock2 (21–24), which are known to be present in granulocytes. The dominant-interfering mutants were transiently coexpressed with CEACAM3 in human 293 cells. This cell line does not express any CEACAM family members endogenously, but efficiently internalizes *N. gonorrhoeae* in an Opa_{CEA}-dependent manner after transfection with CEACAM3 (15). In agreement with previous studies, antibiotic protection assays revealed that following 1 h of infection, CEACAM3-transfected cells harbored numerous intracellular bacteria, whereas control transfected cells were not able to internalize the Opa_{CEA}-expressing gonococci (Fig. 1*A*). When cells were cotransfected with CEACAM3 and dn variants of the Rac GEFs Vav1, SWAP70, ELMO1, or Dock2, only Vav-dn significantly reduced CEACAM3-mediated uptake of gonococci (Fig. 1*A*). Expression analysis of the *myc*-tagged constructs revealed that all dn GEF isoforms were expressed in the transfected 293 cells (Fig. 1*B*). However, Vav-dn was expressed at lower levels compared with the other dn GEFs (Fig. 1*B*). Indeed, increasing the dosage of Vav-dn (Fig. 1*D*) led to further decreases in CEACAM3-dependent internalization (Fig. 1*C*), suggesting that Vav might be involved in CEACAM3-initiated signaling and bacterial uptake.

RNA interference-mediated silencing of Vav interferes with CEACAM3-mediated bacterial uptake and Rac stimulation

To further corroborate a role for this GEF in CEACAM3-initiated signaling we used a well characterized model of stable CEACAM3 expression in HeLa cells. These cells exhibit CEACAM3 phosphorylation and CEACAM3-mediated phagocytosis in response to Opa_{CEA}-expressing bacteria (14, 16). Importantly, siRNA-mediated silencing of Vav2 expression, the Vav isoform predominating in HeLa and 293 cells, not only reduced Vav protein levels to $<$10% of the normal levels, but also severely impaired the uptake of Opa_{CEA}-expressing gonococci (Fig. 2*A*). A control siRNA oligonucleotide directed against firefly luciferase did not compromise Vav protein levels and only marginally reduced CEACAM3-mediated bacterial uptake (Fig. 2*A*). Moreover, siRNA targeting of Vav expression, but not the luciferase-directed control siRNA, also reduced the levels of Rac GTP loading in response to bacterial infection in CEACAM3-expressing HeLa cells (Fig. 2*B*). These results are in line with the idea that Vav participates in CEACAM3-initiated phagocytosis and in particular contributes to CEACAM3-triggered GTP-loading of Rac.

Vav1/2-deficient cells do not show CEACAM3-mediated internalization

To rule out the possibility that Vav-dn expression or the Vav-directed siRNA interfered with the activity of other cellular GEFs,

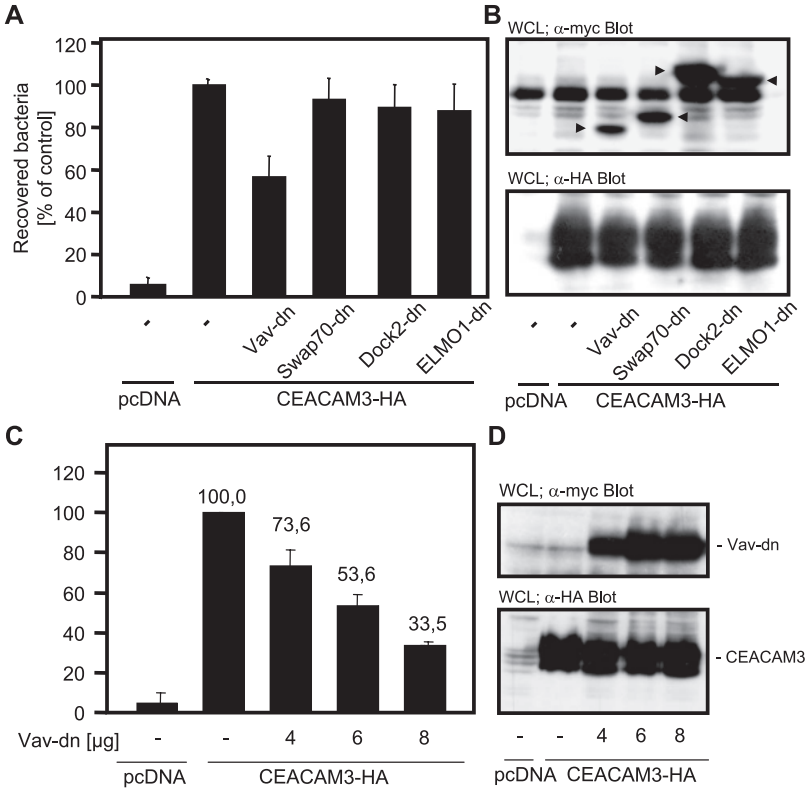

FIGURE 1. The GEF Vav is involved in CEACAM3-mediated internalization of Opa_{CEA}-expressing gonococci. *A*, 293 cells were cotransfected with an empty control vector (pcDNA) or HA-tagged CEACAM3 and the indicated dn versions of the human GEFs Vav1, SWAP70, Dock2, and ELMO1. Samples were infected for 1 h with Opa_{CEA}-expressing gonococci and the number of internalized bacteria was determined by a gentamicin protection assay. Results represent mean ± SD of three independent experiments done in triplicate and show the percentage of recovered bacteria in relation to CEACAM3-expressing cells in the absence of a dn GEF. *B*, Samples in *A* were lysed and the whole cell lysate (WCL) was separated by SDS-PAGE. Expression of the Myc-tagged dn GEFs (arrowheads) and CEACAM3 was confirmed by Western blotting with a mAb against the Myc-tag (*top*) or against the HA-tag (*bottom*). *C*, 293 cells were cotransfected with the indicated amounts of cDNA encoding Vav-dn and CEACAM3-HA or transfected with an empty control vector (pcDNA), respectively. Samples were infected with Opa_{CEA}-expressing gonococci for 1 h and applied in a gentamicin assay. Data show the mean ± SD of two independent experiments done in triplicate in percentage relative to CEACAM3-expressing cells in the absence of Vav-dn. *D*, Samples in *C* were lysed and the whole cell lysate (WCL) was separated by SDS-PAGE. Expression of the *myc*-tagged Vav-dn and HA-tagged CEACAM3 was confirmed by Western blotting with a mAb against the *myc*-tag (*top*) or against the HA-tag (*bottom*), respectively.

we investigated whether genetic ablation of Vav isoforms compromises CEACAM3-mediated bacterial uptake. To this end, primary fibroblast were isolated from Vav1/Vav2 double-knockout mouse embryos and immortalized with SV40 large T Ag (Vav1/$2^{-/-}$ cells). These cells were cotransfected with CEACAM-RFP and HA-tagged Vav1 or the empty control vector. Upon infection with Opa_{CEA}-expressing gonococci, Vav1/$2^{-/-}$ cells re-expressing Vav were able to internalize the bacteria (Fig. 3*A*). In contrast, intracellular bacteria were not observed in CEACAM3-positive Vav1/$2^{-/-}$ cells (Fig. 3*A*). Vav re-expression in Vav1/$2^{-/-}$ cells was confirmed by Western blotting (Fig. 3*B*). These results corroborate the idea that Vav is the critical GEF linking CEACAM3 engagement with the phagocytosis of CEACAM-binding microorganisms.

Vav associates with CEACAM3 upon bacterial binding

To investigate a possible recruitment of Vav to the site of bacterial CEACAM3 engagement, 293 cells were cotransfected with constructs encoding HA-tagged Vav2 and CEACAM3-RFP. Importantly, upon infection of the cells with fluorescein-labeled Opa_{CEA}-expressing gonococci, Vav2 colocalized with CEACAM3 at the sites of bacterial attachment (Fig. 4*A*). Intracellular bacteria were not associated with CEACAM3 or Vav2 suggesting that Vav is transiently recruited to the sites of CEACAM3 clustering during internalization (Fig. 4*A*). To further analyze whether Vav associates with CEACAM3 in intact cells, we transfected 293 cells with combinations of constructs encoding GFP or GFP-tagged CEACAM3 (CEACAM3-GFP) and HA-tagged Vav2 or an empty control vector (Fig. 4*B*). Upon infection with Opa_{CEA}-expressing *N. gonorrhoeae* and precipitation of CEACAM3-GFP with GFP-directed Abs, HA-Vav was specifically detected in the immunoprecipitates (Fig. 4*B*). In the absence of CEACAM3, the anti-GFP Abs did not precipitate Vav from *Neisseria*-infected cells (Fig. 4*B*). These results indicated that Vav is recruited to activated CEACAM3 and is physically associating with a CEACAM3-containing protein complex.

Vav associates with the phosphorylated ITAM-like sequence of CEACAM3

The ITAM-like sequence is a characteristic feature of the CEACAM3 cytoplasmic domain (4). Mutation of the tyrosine residues contained within this sequence to phenylalanine abrogates tyrosine phosphorylation of the receptor, abolishes CEACAM3-initiated GTP-loading of Rac, and severely interferes with opsonin-independent bacterial uptake (15, 16). In contrast, Vav is the only Rac GEF known to contain a phosphotyrosine-binding SH2 domain that is critical to recruit Vav to sites of cytoskeletal remodeling (Fig. 5*A*) (25). Importantly, the Vav-dn protein that interfered with CEACAM3-mediated bacterial uptake encompasses

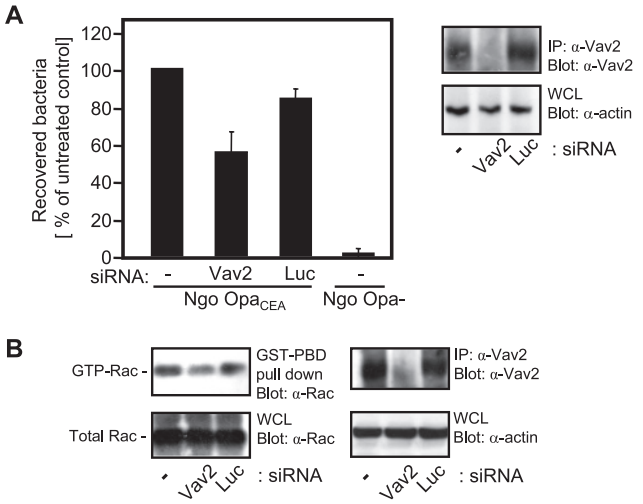

FIGURE 2. Vav depletion by siRNA interference reduces CEACAM3-mediated internalization of Opa_{CEA}-expressing gonococci and Rac stimulation. *A*, HeLa cells expressing CEACAM3 were left untreated or treated for 2 days with Vav2 or luciferase (Luc) siRNA oligonucleotides. Cells were infected for 1 h with Opa_{CEA}-expressing gonococci (Ngo Opa_{CEA}) or nonopaque gonococci (Ngo Opa−) and processed in a gentamicin protection assay. Data represent mean ± SD of recovered bacteria from three independent experiments done in triplicate. In parallel, cells were lysed and Vav2 protein was immunoprecipitated using anti-Vav2 Abs. Western blotting (*right*) confirmed down-regulation of Vav2 upon treatment with Vav2-directed siRNA (*top*). Similar amounts of the whole cell lysates (WCL) were probed with a rabbit polyclonal anti-β-actin Ab (*bottom*). *B*, Cells transfected as in *A* were infected with Opa_{CEA}-expressing gonococci (Ngo Opa_{CEA}). GTP-loading of the small GTPase Rac was determined by a GST-CRIB pull-down assay (*top left*). Western blotting of whole cell lysates (*bottom left*) with mAb anti-Rac confirmed equal amounts of protein in each sample. siRNA-mediated suppression of Vav protein expression was confirmed as in *A* (*right panels*).

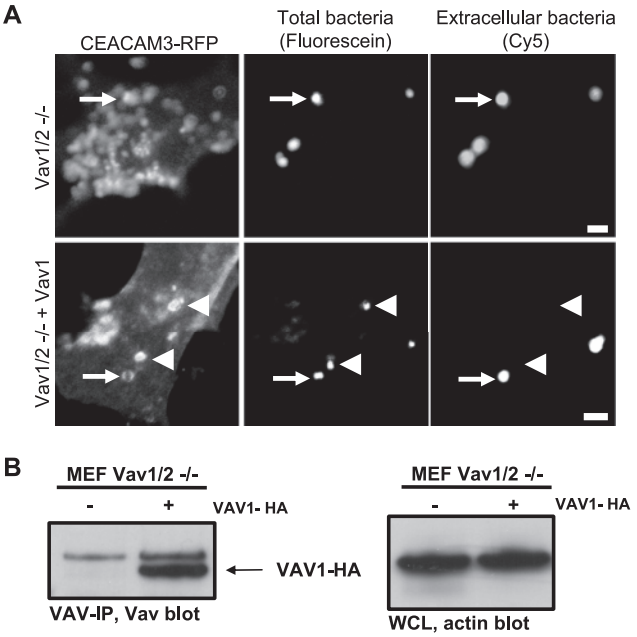

FIGURE 3. Vav1/2-deficient mouse fibroblasts are unable to support CEACAM3-mediated phagocytosis. *A*, Vav1/2-deficient mouse embryo fibroblasts (MEF) were cotransfected with CEACAM3-RFP and the empty control vector (Vav1/2$^{-/-}$) or HA-tagged Vav1 (Vav1/2$^{-/-}$ + Vav1). After infection with fluorescein-labeled Opa_{CEA}-expressing gonococci, infected cells were stained in the absence of permeabilization with Abs directed against gonococci and Cy5. Extracellular bacteria are stained by both fluorescein and Cy5 (small arrows), whereas intracellular bacteria selectively stain for fluorescein (arrowhead). Scale bar represents 5 μm. *B*, Vav was immunoprecipitated from the cell population used in *A* with a polyclonal anti-Vav Ab and after separation by SDS-PAGE, analyzed by Western blotting with the anti-Vav Ab (*left*). Equal amounts of protein were demonstrated in the samples (*right*) by probing with anti-actin Abs.

the SH2 domain prompting us to investigate whether Vav-dn directly associates with phosphorylated CEACAM3. Accordingly, we generated a GST-fusion of Vav-dn and expressed it in *E. coli*. Though the resulting fusion protein was sensitive to proteolytic cleavage, sufficient amounts could be purified from bacterial lysates (Fig. 5*C*). To analyze whether Vav-dn binds to CEACAM3 in a phosphotyrosine-dependent manner, 293 cells were transfected with CEACAM3 together or not with a constitutive active Src family kinase (v-Src) and lysates were prepared from uninfected cells. CEACAM3 was expressed at equal levels in both lysates; however, under these conditions tyrosine phosphorylation of CEACAM3 was only observed in the presence of v-Src (Fig. 5*B*). Pull-down assays with GST-Vav-dn from these lysates demonstrated that the C-terminal part of Vav encompassing the SH2 domain was able to bind to tyrosine phosphorylated CEACAM3, but not unphosphorylated CEACAM3 (Fig. 5*C*). In contrast, GST alone did not bind to CEACAM3 irrespective of the phosphorylation status (Fig. 5*C*). Tyrosine phosphorylated CEACAM3 was also precipitated by a GST-fusion of the isolated Vav SH2 domain, but not by the SH2 domain of the adaptor protein SLP76 (Fig. 5*D*). These results demonstrate that the Vav SH2 domain promotes a specific interaction with the phosphorylated ITAM-like sequence of CEACAM3.

Vav directly binds to phosphorylated Tyr^{230} within the CEACAM3 cytoplasmic domain

To further investigate which tyrosine residue within the CEACAM3 ITAM-like sequence is responsible for Vav-dn binding, 293 cells were cotransfected with v-Src and the empty control vector, CEACAM3 (CEACAM3 WT), or several CEACAM3 mutants (Fig. 6*A*, *lower panel*). These variants of CEACAM3 either harbored phenylalanine substitutions at single tyrosine residues within the ITAM-like sequence (CEACAM3 Y230F or CEACAM3 Y241F), substitutions at both tyrosine residues (CEACAM3 YY-FF), or lacked the complete cytoplasmic domain (CEACAM3 ΔCT). Whereas GST-Vav-dn was able to precipitate CEACAM3 WT as well as CEACAM3 Y241F, mutation of tyrosine residue Tyr^{230} abolished association between CEACAM3 and the C-terminal part of Vav (Fig. 6*A*, *upper panel*). To analyze whether the binding of Vav to the CEACAM3 cytoplasmic domain might be direct or whether this association requires additional adaptor functions, we tested the binding of these two molecules in the absence of further cellular components. Accordingly, a peptide spot membrane was generated that encompassed 15-mer peptides surrounding each of the two tyrosine residues within the CEACAM3 ITAM-like sequence (Fig. 6*B*). Each peptide was represented in the unphosphorylated form (Y), the phosphorylated form (pY), and the phenylalanine-substituted form (F). Importantly, GST-Vav-dn bound to a synthetic phospho-peptide spanning CEACAM3 tyrosine residue Tyr^{230}, whereas there was no binding to the same peptide in the unphosphorylated or the phenylalanine-substituted forms (Fig. 6*C*). This in vitro association was highly specific because Vav-dn did not bind to a synthetic peptide spanning CEACAM3 tyrosine residue Tyr^{241} in the

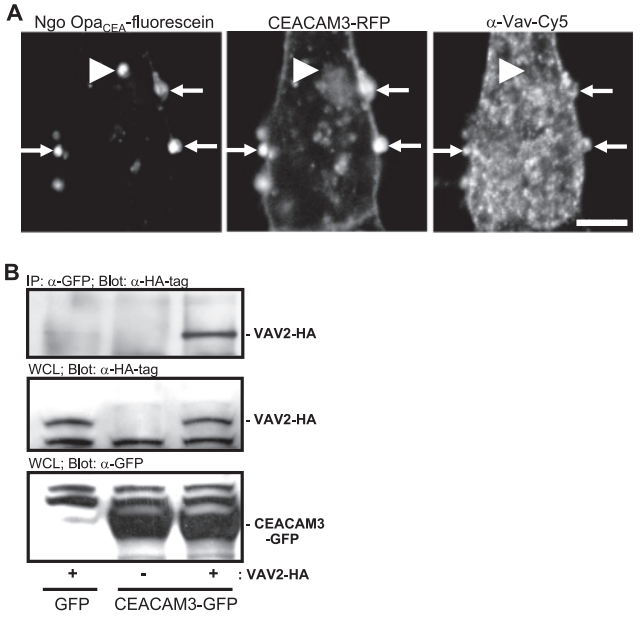

FIGURE 4. Vav associates with CEACAM3. *A*, 293 cells were cotransfected with HA-tagged Vav2 and CEACAM3-RFP and then infected with fluorescein-labeled Opa$_{CEA}$-expressing gonococci. One hour after infection, cells were fixed and stained with anti-Vav Abs and Cy5-coupled secondary reagents (α-Vav-Cy5), before the samples were analyzed by confocal microscopy. Scale bar represents 10 μm. Recruitment of both CEACAM3 and Vav to adherent bacteria is marked by arrows. Intracellular bacteria appear not to be associated with CEACAM3 or Vav (arrowhead). *B*, 293 cells were cotransfected as indicated with plasmids encoding GFP or CEACAM3-GFP and HA-tagged Vav2. Cells were infected with Opa$_{CEA}$-expressing gonococci, lysed, and subjected to immunoprecipitation with anti-GFP Ab. Precipitates were analyzed by Western blotting with anti-HA-tag Ab (*upper panel*). Expression of Vav2-HA (*middle panel*) and CEACAM3-GFP (*bottom panel*) was confirmed in the respective whole cell lysates (WCL) by mouse monoclonal anti-HA-Ab or anti-GFP Ab, respectively.

phosphorylated or in the unphosphorylated form (Fig. 6*C*). Furthermore, there was no association between GST alone and any of the tested peptides detectable (Fig. 6*C*). Together, these results demonstrate that there is a direct and specific interaction between the Vav SH2 domain and the phosphorylated tyrosine residue Tyr^{230} within the ITAM-like sequence of CEACAM3.

Vav is involved in opsonin-independent phagocytosis of CEACAM-binding pathogens by primary granulocytes

To finally investigate whether CEACAM3-initiated signaling in primary granulocytes is also dependent on Vav function, we took advantage of TAT-mediated protein transduction in freshly isolated human granulocytes. Therefore, TAT-fusion proteins of VAV-dn as well as SWAP70-dn were expressed and purified from *E. coli* (Fig. 7*A*). Human granulocytes were preincubated or not with 2 μM of these proteins for 15 min before infection with fluorescein-labeled *N. gonorrhoeae*. In the absence of opsonins, nonopaque gonococci are barely phagocytosed by primary granulocytes, whereas Opa$_{CEA}$-expressing *N. gonorrhoeae* are efficiently internalized within 15 min after infection (Fig. 7*A*) (15). Interestingly, treatment of the phagocytes with TAT-SWAP70-dn did not impair their ability to internalize opaque gonococci compared with untreated control cells (Fig. 7*A*). In contrast, preincubation of the granulocytes with TAT-Vav-dn severely decreased the uptake of Opa$_{CEA}$-expressing gonococci (Fig. 7*A*). To further corroborate these findings, we infected TAT-protein-treated cells with fluorescein-labeled, Opa$_{CEA}$-expressing gonococci. Following infection for 15 min, the samples were fixed and, in the absence of cell permeabilization, extracellular bacteria were selectively marked by anti-*N. gonorrhoeae* Abs and Cy5-conjugated secondary Abs. In addition, TAT-fusion proteins were detected after cell permeabilization using Abs against the HA-tag and Cy-3-conjugated secondary Abs. Confocal microscopy revealed that cells transduced with TAT-SWAP70-dn contained numerous intracellular bacteria (Fig. 7*B*, arrowhead), characterized by their exclusive fluorescein-staining, as well as some cell-associated,

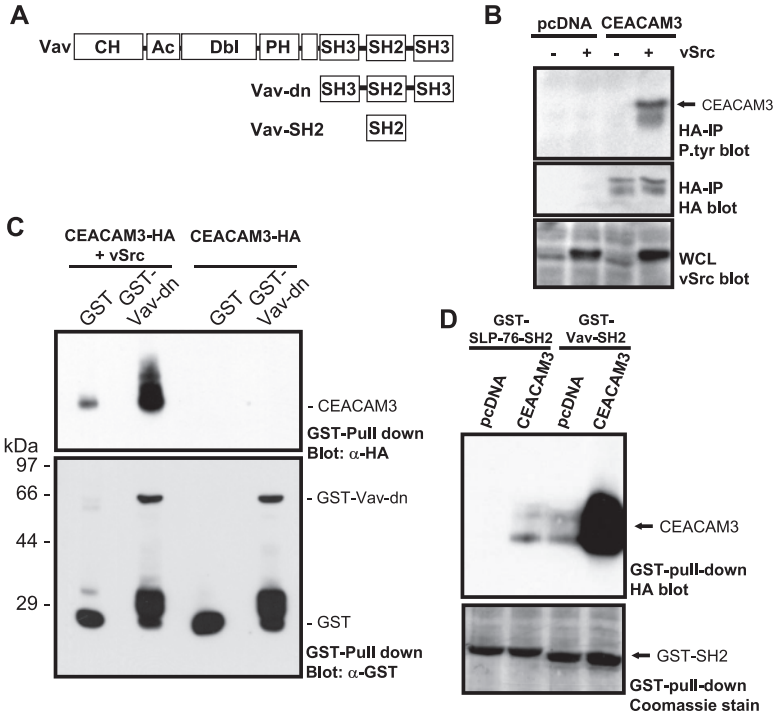

FIGURE 5. The Vav SH2 domain associates with the phosphorylated ITAM-like sequence of CEACAM3. *A*, Schematic representation of the domain structure of Vav, Vav-dn, and Vav SH2. CH, calponin homology; Ac, acidic region; Dbl, Dbl homology domain; PH, pleckstrin homology domain; SH3, Src homology 3 domain. *B*, 293 cells were transfected with the empty control vector (pcDNA) or CEACAM3-HA and cotransfected or not with v-Src. Upon immunoprecipitation of CEACAM3-HA with HA-tag (HA-IP)-directed mAbs, tyrosine phosphorylation of CEACAM3 was detected by Western blotting with phosphotyrosine-specific mAbs (P.tyr blot; *upper panel*). CEACAM3-HA precipitation (*middle panel*) and v-Src expression (*lower panel*) in the respective samples was also verified by Western blotting with the indicated mAbs. *C*, 293 cells were cotransfected as in *B*. Whole cell lysates (WCLs) were subjected to a pull-down assay with 10 μg of GST-Vav-dn or GST, and the GST proteins visualized by Coomassie staining of the membrane (*bottom*). Coprecipitated CEACAM3 was detected by anti-HA Ab (*top*). *D*, 293 cells were cotransfected with v-Src and CEACAM3-HA or the empty control vector (pcDNA), respectively. Cell lysates were incubated with 10 μg of GST-SLP76-SH2 or GST-Vav-SH2 and the precipitates analyzed by blotting with anti-HA Ab (*top*). Equal amounts of the recombinant GST proteins in the precipitates were confirmed by Coomassie-staining of the membrane (*bottom*).

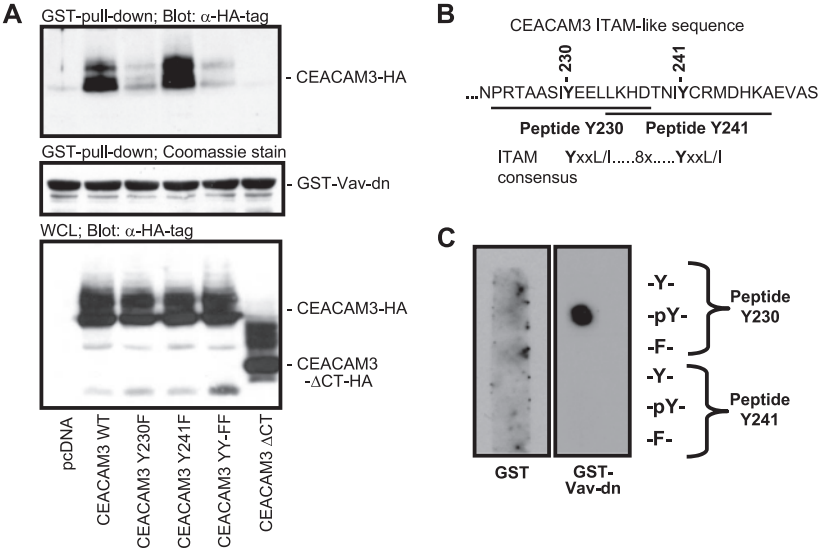

FIGURE 6. Vav directly binds to phosphorylated Tyr^{230} of CEACAM3. *A*, 293 cells were cotransfected with the empty control vector (pcDNA) or the indicated HA-tagged CEACAM3 constructs and v-Src. Whole cell lysates (WCL) were analyzed for equal expression of CEACAM3 constructs by anti-HA Ab (*lower*). Lysates were subjected to a pull-down assay with GST-Vav-dn and the GST-fusion protein was visualized by Coomassie staining of the membrane (*middle*). CEACAM3 variants coprecipitating with GST-Vav-dn were detected by anti-HA Ab (*upper*). *B*, Primary structure of the ITAM-like sequence within the CEACAM3 cytoplasmic domain (CT). For comparison, the ITAM consensus sequence is aligned below. *C*, Peptide spot membranes harboring synthetic 15-mer peptides surrounding the indicated tyrosine residues of the CEACAM3 cytoplasmic domain (as indicated in *B*) in the unphosphorylated (Y), the tyrosine-phosphorylated (pY), or the phenylalanine-substituted (F) form were probed with GST or GST-Vav-dn. Bound GST-fusion proteins were detected with anti-GST Ab.

extracellular bacteria that stained positive for both fluorescein and Cy5 (Fig. 7*B*, small arrow). In contrast, no intracellular bacteria were detected in TAT-Vav-dn transduced granulocytes and all cell-associated bacteria were clearly highlighted with both fluorescein and Cy5 (Fig. 7*B*, small arrows) proving their extracellular location. Together, these data further support the idea that Vav links CEACAM3 engagement with Rac GTP loading and demonstrate that this functional connection is critical for opsonin-independent CEACAM-mediated phagocytosis of Opa_{CEA}-expressing gonococci by primary human granulocytes.

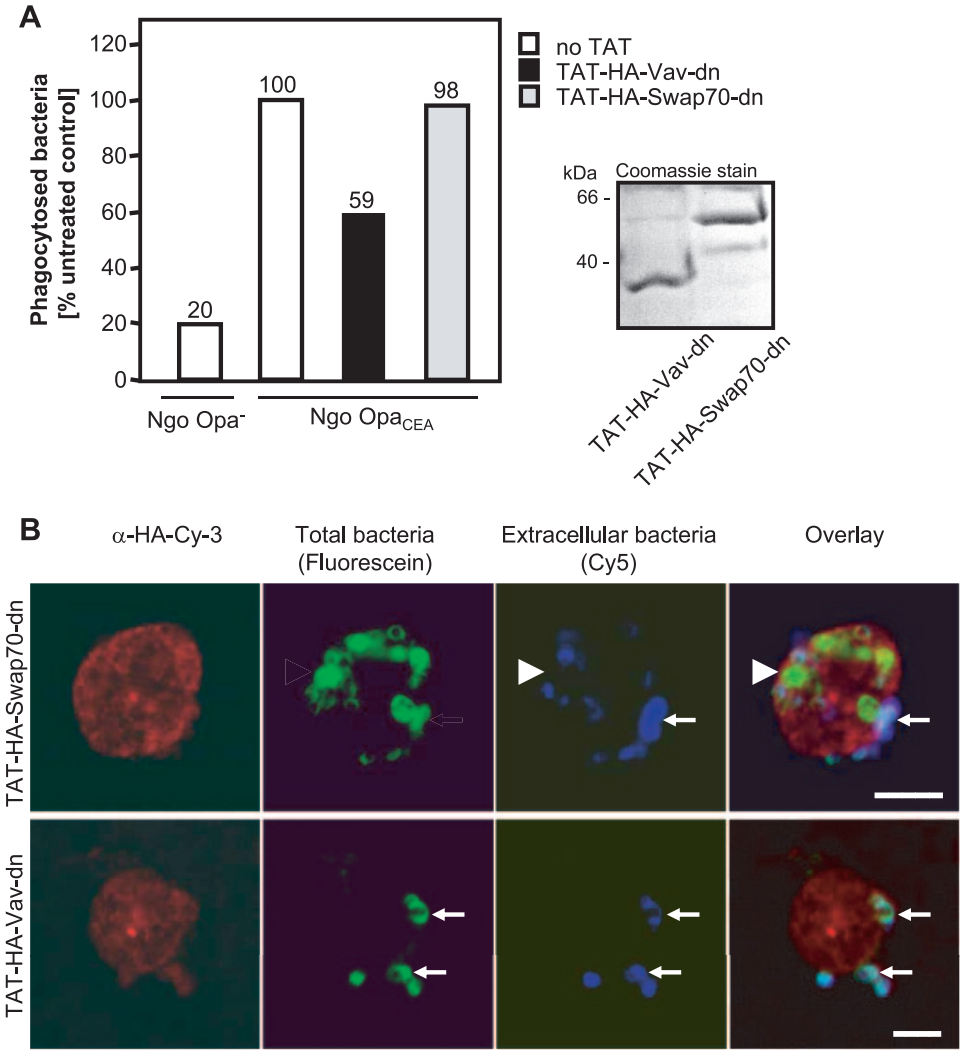

FIGURE 7. Vav is required for CEACAM3-mediated phagocytosis by primary human granulocytes. *A*, Isolated human neutrophils were left untreated or preincubated for 1 h at 37°C with 2 μM TAT-HA-Vav-dn or TAT-HA-SWAP70-dn before infection with fluorescein-labeled non-opaque (Ngo Opa^-) or Opa_{CEA}-expressing gonococci (Ngo Opa_{CEA}) for 15 min at 37°C. Phagocytosis was determined by flow cytometry. Data represent the uptake index in relation to untreated cells and are derived from a representative experiment. Similar results were obtained with granulocytes isolated from three different donors. A Coomassie-stained gel (*right*), where 5 μg of the indicated purified TAT-fusion proteins were loaded. *B*, Isolated human granulocytes were pretreated with either TAT-HA-Vav-dn or TAT-HA-SWAP70-dn as in *A* before infection with fluorescein-labeled, Opa_{CEA}-expressing gonococci. Samples were fixed and differentially stained for extracellular (arrow, Cy5- and fluorescein-positive) and intracellular bacteria (arrowhead, fluorescein-positive only). The transduced TAT-fusion proteins were visualized after cell permeabilization by using a monoclonal anti-HA-tag Ab and a Cy3-conjugated rabbit anti-mouse Ab (α-HA-Cy3).

Discussion

CEACAM3 (formerly designated CGM1a or CD66d) is a specialized member of the CEACAM family that is exclusively expressed on granulocytes (17). So far, no homologs of CEACAM3 have been detected in species other than humans, suggesting that this receptor is a recent invention in primate evolution (4). In contrast to other CEACAM family members, CEACAM3 does not engage in homophilic or heterophilic interactions with other CEACAMs and does not contribute to cell-cell adhesion (26). Furthermore, no endogenous ligands of CEACAM3 have been identified at present. However, this receptor is responsible for the efficient opsonin-independent recognition and elimination of a number of human-specific, Gram-negative bacterial pathogens (15). These findings suggest that CEACAM3 represents a specialized component of the human innate immune defense against a subgroup of bacterial pathogens that exploit CEACAMs present on mucosal surfaces for successfully colonizing their human host.

In this study, we provide evidence that CEACAM3-mediated, opsonin-independent phagocytosis of human pathogens depends on a direct physical interaction between a tyrosine phosphorylated residue within the CEACAM3 ITAM-like sequence and the Rac GEF Vav. This tyrosine kinase-regulated protein-protein interaction directly couples CEACAM3-mediated recognition of bacteria with the stimulation of the small GTPase Rac that is critical for several effector functions of human granulocytes, such as actin cytoskeleton rearrangements leading to phagocytosis and the generation of reactive oxygen derivatives by the NADPH oxidase (27, 28). In this respect, the recruitment of Vav to tyrosine phosphorylated CEACAM3 provides a molecular explanation for the efficient opsonin-independent phagocytosis and elimination of CEACAM3-binding bacteria by the human innate immune defense.

Vav connects multiple immunoreceptors with actin cytoskeleton dynamics

Vav stands out from other members of the Dbl homology family of GEFs, as it encompasses a SH2 domain that allows Vav to connect to phosphorylated tyrosine residues in a number of proteins (29). Furthermore, Vav GEF activity itself is regulated by tyrosine phosphorylation enabling Vav to couple PTK signaling pathways to Rho GTPase stimulation (30). Although in vitro Vav seems to be able to exert GEF activity toward multiple Rho family members, the preferred substrate for Vav appears to be the small GTPase Rac (31). For example, upon stimulation of the FcεRI found on basophils and mast cells, Vav activity is directed toward Rac, but does not affect other small GTPases such as Cdc42, RhoA, or Ras (32). Similarly, during FcγR-mediated phagocytosis Vav is exclusively coupled with guanine nucleotide exchange on Rac, but not on Cdc42 (33). Our data provide additional support to the notion that Vav acts as a specific Rac GEF in vivo, as CEACAM3-mediated phagocytosis depends on Rac, but not on Cdc42 GTP loading, and contact of human phagocytes with Opa_{CEA}-expressing gonococci leads to rapid Rac, but not Cdc42 stimulation (12, 15).

Molecular interactions mediated by the Vav SH2 domain and the CEACAM3 ITAM-like sequence

In ITAM-initiated signaling by the TCR, by the BCR, by FcεR1, or by FcγR, Vav is recruited to the activated receptor complex by an indirect mechanism that involves Vav SH2 domain binding to the tyrosine phosphorylated adaptor molecules SLP76/SLP65 (BLNK) or either one of the cytoplasmic PTKs Zap70 or Syk (29, 34). Additional adaptor molecules such as linker for activation of T cells or Gads can then be involved in linking Vav-associated molecules with the immunoreceptor complex (35). In contrast, the Vav SH2 domain binds directly to the cytoplasmic ITAM-like sequence of CEACAM3 bypassing the requirement for any accessory adaptor proteins. Such a kind of short-wiring might provide fast signal propagation from the receptor; however, it might reduce the ability to fine-tune and regulate CEACAM3-initiated phagocytosis.

Recognition sites for the Vav SH2 domain have been characterized in Zap70 (Tyr^{313}), Syk (Tyr^{341}), and SLP76 (Tyr^{112} and Tyr^{128}), in which the critical tyrosine residues are embedded within a canonical YESP motif (36). The Vav SH2 domain has also been shown to bind to a YEEP sequence in the cytoplasmic domain of CD19 (37). In addition, our data support the view that the Vav SH2 domain is able to associate with the YEEL sequence surrounding Tyr^{230} of CEACAM3. It is interesting to note that the second tyrosine residue within the CEACAM3 cytoplasmic domain ITAM-like sequence, Tyr^{241}, corresponds to a YxxM motif. Such a motif has been found in other instances to serve as high affinity docking site for the SH2 domains of the regulatory p85 subunit of PI3K (38). Indeed, PI3K activity is required for CEACAM3-initiated bacterial uptake and a product of PI3K activity, phosphatidylinositol-3,4,5-trisphosphate has been detected in the vicinity of bacteria-bound CEACAM3 (39). Importantly, phosphatidylinositol-3,4,5-trisphosphate allows maximum activation of the Vav GEF function by binding to the Vav pleckstrin homology domain (40). Therefore, the phosphorylated ITAM-like sequence of CEACAM3, with its particular protein-protein interactions, might spatially bring together both an activator of GEF activity (PI3K) as well as the specific GEF itself (Vav) for a locally confined and rapid stimulation of the small GTPase Rac.

Vav short-wires CEACAM3 phosphorylation with Rac GTP loading for efficient bacterial phagocytosis and elimination

It has to be considered that in granulocytes CEACAM3 is coexpressed with other CEACAM family members such as CEACAM1 and GPI-anchored CEACAM6 that both recognize Opa_{CEA}-expressing neisseriae. In particular, CEACAM1 has been described to function as a negative regulator of signaling events in the context of TCR stimulation and NK cell signaling (41–43). This inhibitory function is presumably due to a functional ITIM within the cytoplasmic domain of the most common isoform of CEA CAM1 (44, 45). Therefore, granulocyte contact with the multivalent Opa_{CEA}-expressing gonococci should engage several CEACAM variants including CEACAM1, CEACAM3, and CEACAM6. Whereas signal transduction from GPI-anchored CEACAM6 has not been characterized so far, CEACAM-binding pathogens should simultaneously trigger CEACAM3-initiated stimulation of Src PTKs, Vav, and Rac as well as CEACAM1-mediated inhibitory signaling events. It is interesting to speculate that the direct association of Vav with phosphorylated Tyr^{230} of CEACAM3 and the resulting fast kinetics of Rac GTP-loading provide a molecular rationale for the fact that CEACAM3 functions as a highly effective phagocytic receptor in the presence of CEACAM1-initiated negative signaling. Furthermore, the complex formation between CEACAM3 and Vav could help explain previous observations that the kinase Syk, a canonical element in ITAM-initiated signaling, is not stimulated upon gonococcal contact with human phagocytes and is not essential for CEA CAM3-mediated phagocytosis (12) (S. Gray-Owen, personal communication). Taken together, the short-wiring of the CEACAM3 ITAM-like motif with Rac stimulation via direct Vav recruitment might represent a specific adaptation of the human innate immune system. In this way, CEACAM1-initiated negative signaling could be excluded from interfering with CEACAM3-mediated phagocytosis, thereby allowing the opsonin-independent elimination of CEA CAM-binding pathogens by human granulocytes.

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Disclosures

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