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Tyrosine Phosphorylation of Carcinoembryonic Antigen-related Cell Adhesion Molecule 20 and Its Functional Role

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Carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 20 is an immunoglobulin-superfamily transmembrane protein that contains an immunoreceptor tyrosine-based activation motif (ITAM) in its cytoplasmic region. However, the mechanism for tyrosine phosphorylation of, or the physiological function of, this protein remains largely unknown. Here we have shown that CEACAM20 is indeed tyrosine-phosphorylated by either treatment with pervanadate or forced expression of c-Src. In addition, Tyr^{522}, Tyr^{559} or Tyr^{570}, the latter two of which are within the ITAM, is likely important for such tyrosine phosphorylation. Forced expression of Myc-tagged wild-type CEACAM20 promoted the phagocytic activity of cultured cells for microbeads coupled with anti-Myc antibodies. By contrast, such phagocytic activity was markedly reduced when a mutant form of CEACAM20, in which Tyr^{559} and Tyr^{570} were substituted with phenylalanine, was expressed. Furthermore, the CEACAM20-mediated phagocytic activity was markedly prevented by the treatment with an inhibitor for either Src family kinases (SFKs), Syk, phosphoinositide 3-kinase (PI3K) or phospholipase C-γ (PLCγ). Inhibition of actin polymerization by Cytochalasin D significantly inhibited the CEACAM20-mediated phagocytosis. These results thus suggest that tyrosine phosphorylation of CEACAM20 likely promotes phagocytic activity of the cells. The CEACAM20-mediated phagocytic activity requires the activation of SFKs, Syk, PI3K or PLCγ.

The carcinoembryonic antigen-related cell adhesion molecule (CEACAM) family is a member of the immunoglobulin (Ig) superfamily and consists of 12 or 8 family members in human or mouse, respectively [10,11]. Most members of this family, with the notable exceptions such as CEACAM16 and CEACAM10, are characterized by a single N-terminal

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Ig variable (IgV)-like domain (N-domain) and a variable number of Ig constant (IgC)-like domains in their extracellular regions. From their primary amino acid sequences, some of members are predicted to be membrane-bound, or the others are thought to be glycosylphosphatidylinositol-anchored or secreted [10,11,24]. Each member of CEACAMs manifests specific expression pattern. In particular, CEACAM1 is predominantly expressed in epithelial, endothelial and immune cells [7]. The expression of CEACAM3 is specifically found in human granulocytes [7]. Compelling evidence suggests that members of the CEACAM family are crucial modulators of many cellular functions, such as cell growth, apoptosis, cell-cell adhesion and immune responses [7,10,11]. In addition, some family members such as CEACAM1, -3, -5 and -6, act as receptors that interact with bacterial or viral pathogens through their N-domain [7].

CEACAM1 consists of a variety of alternative splicing isoforms in human and mouse [7,9]. CEACAM1-L, a major isoform of CEACAM1, comprises extracellular Ig-like domains with a single N-domain and a cytoplasmic domain with two immunoreceptor tyrosine-based inhibitory motifs (ITIMs). This isoform is thought to be tyrosine phosphorylated at the ITIMs by Src family kinases (SFKs), thereby negatively regulating EGF receptor-, B cell receptor- or T cell receptor-mediated signaling [1,12,16]. In addition, interaction of CEACAM1-L ectodomain with pathogens also promotes tyrosine phosphorylation of the ITIMs, thereby preventing the Toll-like receptor 2-mediated antibacterial responses [19]. CEACAM3 contains a single N-terminal IgV-like extracellular domain as well as a single ITAM-like motif in its cytoplasmic domain. Interaction of the CEACAM3 ectodomain with pathogens induces tyrosine phosphorylation by SFKs of CEACAM3 ITAM-like motif and it promotes phagocytosis and elimination by neutrophils of pathogens [4]. Thus, phosphorylation of the ITIMs or the ITAM is key events for the function of CEACAM1 or CEACAM3, respectively.

Ceacam20 gene is found in mammals including human, mouse and rat [10]. Of interest is that mouse CEACAM20 mRNA is expressed in the limited tissues such as the small intestine and colon [24]. Human or mouse CEACAM20 possesses four IgC-like domains without an N-terminal IgV-like domain in its extracellular region [10,24]. Moreover, CEACAM20 also possesses a single ITAM in its cytoplasmic region. However, it remains unknown whether the ITAM of CEACAM20 is phosphorylated or not. In addition, the physiological function of CEACAM20 has never been investigated. Here we have shown that CEACAM20 indeed undergoes phosphorylation at its ITAM and that such tyrosine phosphorylation is likely important for the CEACAM20-mediated phagocytic activity of cultured cells.

MATERIALS AND METHODS

Antibodies and reagents

Mouse monoclonal antibodies (mAbs) to phosphotyrosine (4G10) and to v-Src were from Merck Millipore (Billerica, MA). A mouse mAb to c-Myc (9E10) was from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse mAb to β-tubulin was from Sigma-Aldrich (St. Louis, MO). Cy3-conjugated goat polyclonal antibodies (pAbs) to mouse IgG, and horse radish peroxidase-conjugated goat pAbs to rat IgG and to mouse IgG were from Jackson ImmunoResearch (West Grove, PA). Alexa350-conjugated goat pAbs to rabbit IgG were from Invitrogen (Carlsbad, CA). Rabbit pAbs specific for mouse CEACAM20 were generated against glutathione S-transferase (GST) fusion proteins containing the cytoplasmic region (amino acids 485-577) of CEACAM20 (GST-CEACAM20-CP). Rabbits were injected with GST-CEACAM20-CP proteins, and the resulting pAbs to CEACAM20 were purified from serum by the use of columns containing GST or GST-CEACAM20-CP

proteins immobilized on CNBr-Sepharose (GE Healthcare; Waukesha, WI) as described previously [14]. PP2, PP3, LY294002, BAY61-3606 and U73122 were from Merck Millipore. Cytochalasin D was from Wako (Osaka, Japan). Poly-L-lysine was from Sigma-Aldrich.

Expression vectors

To construct an expression vector for wild-type mouse CEACAM20 (CEACAM20 WT), a full-length cDNA of mouse CEACAM20 was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) with total RNA from mouse intestine (C57BL/6) and the following primers: forward primer, 5'-GAACTCATGGAGCTTGCTTCC-3', and reverse primer, 5'-TCAGGCTGATGGGGTGATCTT-3'. The resulting PCR products were subcloned into pcDNA3.1 (Invitrogen). For generation of an expression vector for a mutant CEACAM20 ΔCP, which lacks almost the entire cytoplasmic region of CEACAM20 (amino acids 461-577), a cDNA fragment encoding CEACAM20 (amino acids 1-460) was amplified by PCR with mouse CEACAM20 cDNA as the template and the following primers: forward primer, 5'-GAACTCATGGAGCTTGCTTCC-3', and reverse primer, 5'-TCATCAGCGAGTCCATCTGTCCTTTG-3'. The resulting PCR products were subcloned into pcDNA3.1. For construction of expression vectors for CEACAM20-2YF, -3YF and -4YF, in which Tyr^{559} and Tyr^{570} (for 2YF), Tyr^{522}, Tyr^{559} and Tyr^{570} (for 3YF), or Tyr^{495}, Tyr^{522}, Tyr^{559} and Tyr^{570} (for 4YF) were replaced by phenylalanine, respectively (**Fig. 1A**), the mutated cDNAs were generated by PCR-ligation-PCR mutagenesis as described previously [14]. An expression vector for N-terminal Myc-tagged CEACAM20 (Myc-CEACAM20), in which a Myc epitope was inserted between amino acid residues 30 and 31 of mouse CEACAM20, was also generated by PCR-ligation-PCR mutagenesis. A plasmid encoding c-Src was prepared as described [15]. The sequences of all PCR products were verified by sequencing with ABI3100 (Applied Biosystems, Foster City, CA).

Cell culture and transfection

HEK293A cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (Wako) supplemented with 10 % fetal bovine serum and 2 mM L-glutamine. Cells were transfected with expression vectors by the use of Lipofectamine2000 (Invitrogen) in accordance with the manufacturer's instruction. Pervanadate was prepared immediately before the use by mixing 50 mM sodium orthovanadate with 50 mM hydrogen peroxide for 15 min.

Immunoprecipitation and immunoblot

Cells were washed with ice-cold phosphate buffered saline (PBS) and then lysed on ice with TNE buffer [20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1 mM ethylenediamine-N,N,N',N'-tetraacetic acid, 1% TritonX-100] containing 1 mM phenylmethylsulfonyl fluoride, aprotinin (10 μg/ml aprotinin) and 1 mM sodium orthovanadate. The lysates were centrifuged at 17,400 × *g* for 15 min at 4°C, and the resulting supernatants were subjected to immunoprecipitation and immunoblot analysis as described previously [15].

Phagocytosis assay

For preparation of antibody-coupled microbeads, 2 μm fluoresbrite Yellow Green (YG) carboxylate microspheres (Polysciences, Warrington, PA) were incubated with mAbs to Myc

(50 μg/ 1.0×10^9 beads) in the presence of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, a cross-linker, in accordance with the manufacturer's instruction. For phagocytosis assay, transfected cells (2.5×10^5) were replated on cover glasses coated with poly-L-lysine and cultured for 12 h, after which anti-Myc antibody-coupled microbeads (3.5×10^6) were added to the cells and incubated at 37°C for 4 h. Cells were then gently washed with ice-cold PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. For detection of microbeads that were associated with the cells but not internalized, cells were incubated with Cy3-conjugated goat pAbs to mouse IgG before being permeabilized. The cells were then permeabilized and subjected to immunostaining with rabbit pAbs to CEACAM20 and Alexa350-conjugated goat pAbs to rabbit IgG for detection of Myc-CEACAM20-expressing cells. The images of the stained cells or fluorescent microbeads were captured by the use of a differential interference contrast and a fluorescence microscope (BX51, Olympus, Tokyo). The microbeads, which were identified by their YG fluorescence but not stained with Cy3-conjugated pAbs, were thought to be "internalized". Thus, the numbers of the "total" YG–positive microbeads, as well as of YG–positive but Cy3–negative "internalized" beads, were counted for each Myc-CEACAM20-expressing cell. The data were collected from twenty Myc-CEACAM20-expressing cells and averaged.

Statistical analyses

Data are presented as means ± SE and were analyzed by Mann-Whitney *U* test or by Kruskal-Wallis test, and post hoc comparisons were made using Dunn's test. Statistical significance was set at $p < 0.05$.

RESULTS

Tyrosine phosphorylation by pervanadate or c-Src of CEACAM20

The cytoplasmic region of mouse CEACAM20 contains four tyrosine residues, two of which constitute the ITAM (Y^{559}EKL-X_7-Y^{570}CKI)(**Fig. 1A**). To examine whether CEACAM20 indeed undergoes tyrosine phosphorylation, we generated an expression vector for wild-type Myc-tagged mouse CEACAM20 (Myc-CEACAM20 WT), in which the Myc-epitope tag was fused with the N-terminal region of mouse CEACAM20. HEK293A cells were transiently transfected with an expression vector for Myc-CEACAM20 WT, after which the cells were treated with 100 μM pervanadate (PV), a potent inhibitor for protein tyrosine phosphatases, for 5 min. The cells were then lysed and subjected to immunoprecipitation with anti-Myc mAbs. Immunoblot of the immunoprecipitates with anti-phosphotyrosine mAbs showed that CEACAM20 was tyrosine-phosphorylated at the basal condition (**Fig. 1B**). In addition, the extent of tyrosine phosphorylation of CEACAM20 was markedly increased by treatment of cells with PV (**Fig. 1B**). We next examined which tyrosine residues in the cytoplasmic region are important for tyrosine phosphorylation by PV of CEACAM20. Expression vectors for the following four mutant forms of CEACAM20 were generated: 2YF, a mutant contains phenylalanine substitutions for Tyr^{559} and Tyr^{570} at the ITAM; 3YF contains phenylalanine substitutions for Tyr^{522}, Tyr^{559} and Tyr^{570}; 4YF contains phenylalanine substitutions for Tyr^{495} as well as other three tyrosine resides; ΔCP mutant lacks the entire cytoplasmic region (**Fig. 1A**). The cultured HEK293A cells were transfected with expression vectors for WT or other mutant forms of Myc-CEACAM20, after which the cells were treated with PV. The extent of tyrosine phosphorylation of 2YF mutant was markedly reduced, compared with that apparent with WT (**Fig. 1C**). Moreover, tyrosine phosphorylation of 3YF mutant, as well as that of 4YF or of ΔCP mutant, was minimal (**Fig. 1C**). These results suggest that the tyrosine residues, Tyr^{522}, Tyr^{559} or Tyr^{570}, are important

for tyrosine phosphorylation by PV of CEACAM20, although we have not proved yet whether both Tyr^{559} and Tyr^{570} or one of these two tyrosine residues is important for tyrosine phosphorylation by PV of CEACAM20.

SFKs are thought to be important for the phosphorylation of tyrosine residues in the ITAM of either FcγR or CEACAM3, or in the ITIMs of CEACAM1 [3,13,18,22]. We thus next examined the importance of SFKs for promotion of tyrosine phosphorylation of CEACAM20. Indeed, tyrosine phosphorylation of CEACAM20 WT was markedly increased by co-expression with c-Src (**Fig. 1D**). In contrast, the level of tyrosine phosphorylation of 2YF mutant in the cells expressing c-Src was much lower than that of WT (**Fig. 1D**). In addition, tyrosine phosphorylation of 3YF mutant, as well as that of 4F or ΔCP mutant, was undetectable (**Fig. 1D**). Thus, c-Src is likely important for the phosphorylation of three tyrosine residues (Tyr^{522}, Tyr^{559} or Tyr^{570}) at the C-terminus of CEACAM20.

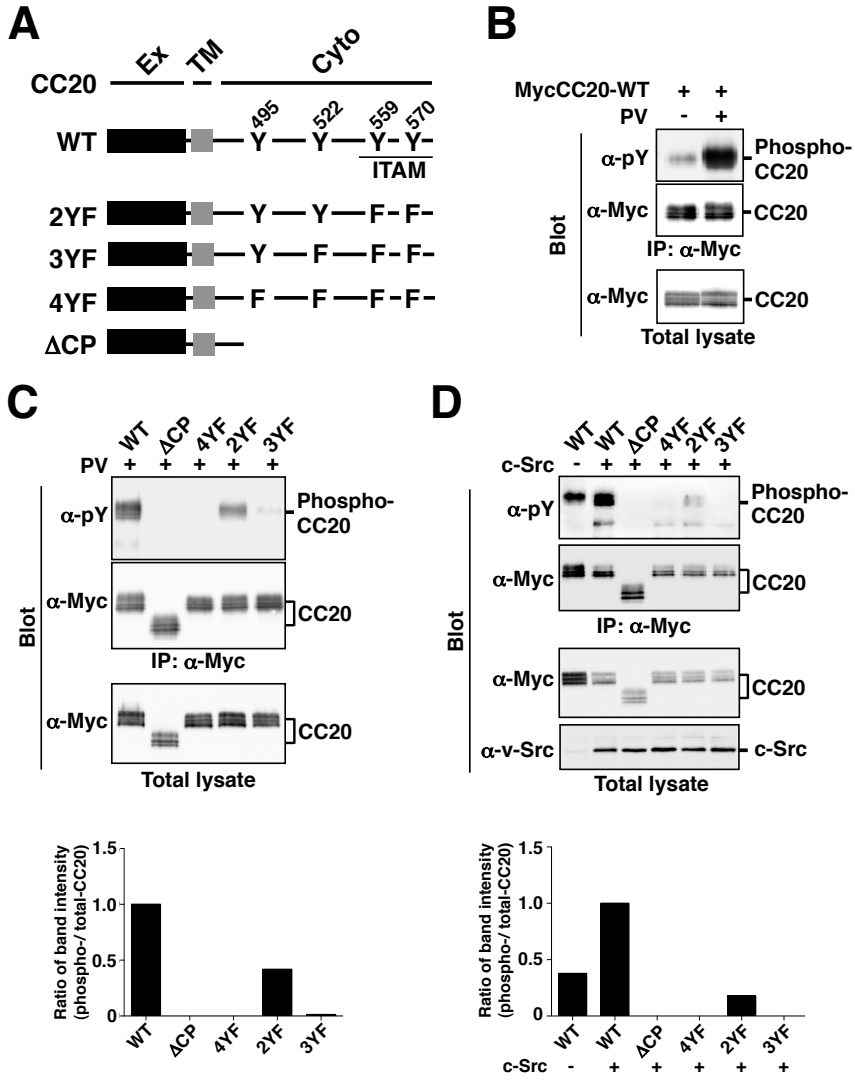

Figure 1. Tyrosine phosphorylation by pervanadate or c-Src of CEACAM20. (**A**) Schematic representation of wild-type or mutants of CEACAM20 (CC20). Numbers indicate amino

acid residues. F, phenylalanine residue; Y, tyrosine residue. Ex, extracellular domain; TM, transmembrane domain; Cyto, cytoplasmic domain; ITAM, immunoreceptor tyrosine-based activation motif. (**B**) HEK293A cells were transfected with an expression vector for Myc-CEACAM20 WT (MycCC20-WT), after which the cells were treated with or without 100 μM of pervanadate (PV) for 5 min. Cell lysates were then subjected to immunoprecipitation (IP) with anti-Myc mAbs (α-Myc), followed by immunoblot analysis with either anti-Myc or anti-phosphotyrosine (pY) mAbs (α-pY). Total cell lysates were also subjected to immunoblot analysis with anti-Myc mAbs to verify the amount of CC20 protein in each cell lysate. (**C**) HEK293A cells were transfected with an expression vector for either MycCC20-WT, -ΔCP, -4YF, -2YF or -3YF, after which the cells were treated and subjected to immunoprecipitation or immunoblot analysis as in (B) (upper panels). Immunoblots were subjected to densitometric analysis, and the ratio of the band intensity for tyrosine-phosphorylated CC20 (phospho-CC20) to that for total CC20 was calculated (lower panel). Data are expressed relative to the corresponding value for cells expressing MycCC20-WT. (**D**) HEK293A cells were transfected with an expression vector for either MycCC20-WT, -ΔCP, -4YF, -2YF or -3YF together with an empty vector or an expression vector for c-Src, after which the cells were subjected to immunoprecipitation or immunoblot analysis as in (B). Total cell lysates were also immunoblotted with either anti-Myc or anti-v-Src mAbs (α-v-Src) to verify the amount of CC20 or c-Src protein in each cell lysate (upper panels). Immunoblots were subjected to densitometric analysis, and the ratio of the band intensity for phospho-CC20 to that for total CC20 was calculated (lower panel). Data are expressed relative to the corresponding value for cells expressing MycCC20-WT and c-Src. Data are representative from three independent experiments (**B, C** and **D**).

Forced expression of wild-type CEACAM20 promotes the phagocytic activity of cultured cells

CEACAM family members, such as CEACAM1 and CEACAM3, are known to directly bind Opa_{CEA}-expressing gonococci through their extracellular domains, and this interaction induces the uptake of the bacteria into the cells such as a human myelomonocytic cell line (JOSK-M) and granulocytes [2,8]. Tyrosine phosphorylation of the ITAM-like motif of CEACAM3 is also shown to be important for the promotion of the phagocytic activity against bacteria [2]. Therefore, we investigated whether CEACAM20 promotes phagocytic activity of cultured cells. To this end, we prepared the fluorescent microbeads (2 μm diameter), which were covalently coupled with anti-Myc mAbs, as a phagocytic target. HEK293A cells were transiently transfected with either an expression vector for Myc-CEACAM20 WT or a control vector, after which the cells were incubated with the anti-Myc mAb-coupled microbeads for 4 h. The resultant cells were then fixed and immunostained with Cy3-conjugated pAbs to mouse IgG (to detect microbeads that bound the anti-Myc mAb) without permeabilization, after which the cells were immunostained with pAbs to CEACAM20 (to identify Myc-CEACAM20-expressing cells) with permeabilization. Thus, the microbeads, which were identified by their fluorescence but not stained with Cy3-conjugated pAbs, were thought to be "internalized". As shown in **Fig. 2**, forced expression of Myc-CEACAM20 WT markedly increased the number of internalized microbeads compared with control cells (mock-transfected cells), suggesting that CEACAM20 likely promotes the phagocytic activity of the cultured cells.

Importance of tyrosine phosphorylation of CEACAM20 for the promotion of phagocytic activity

We next determined whether tyrosine phosphorylation of CEACAM20 is important for the CEACAM20-promoted phagocytic activity. Transfection of HEK293A cells with

expression vectors for either WT or other three YF mutants yielded the similar expression of each CEACAM20 protein (**Fig. 3A**). However, the number of microbeads internalized into WT-expressing cells was much greater than that apparent with cells expressing other mutants (**Fig. 3B**). By contrast, the total numbers of microbeads that were associated with the CEACAM20-expressing cells were comparable between WT and other three mutants (**Fig. 3B**). These results suggest that the replacement of the tyrosine residues, such as Tyr^{522}, Tyr^{559} or Tyr^{570}, by phenylalanine markedly reduces the CEACAM20-promoted internalization of microbeads. Moreover, the numbers of microbeads internalized into the cells were almost comparable between three YF mutants (**Fig. 3B**), suggesting that the phosphorylation of Tyr^{559} or Tyr^{570} is likely important for the CEACAM20-mediated phagocytic activity.

Given that c-Src is important for tyrosine phosphorylation of CEACAM20, we further investigated whether SFKs participate in the CEACAM20-mediated phagocytic activity. PP2, an inhibitor for SFKs, but not DMSO (vehicle) or PP3, an inactive analog of PP2, markedly inhibited the CEACAM20-mediated internalization of the microbeads into the cells (**Fig. 3C**). These results suggest that SFKs are important for the CEACAM20-mediated phagocytic activity.

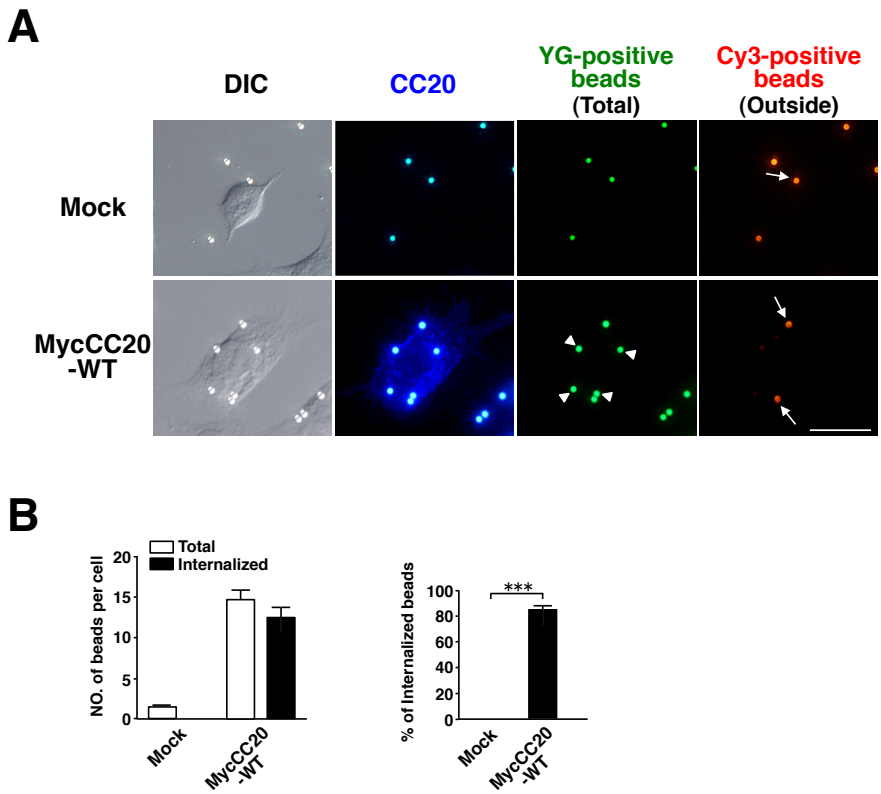

Figure 2. Forced expression of wild-type CEACAM20 promotes the phagocytic activity of cultured cells. (**A**) HEK293A cells were transfected with an empty expression vector (Mock) or an expression vector for MycCC20-WT. Twelve h after transfection, cells were replated on cover glasses coated with poly-L-lysine and cultured for additional 12 h. The cells were then incubated with anti-Myc mAb-coupled microbeads (**Total** YG–positive beads, as indicated by green)(right middle panels) for 4h, after which, cells were fixed and immunostained to identify CC20-expressing cells (**CC20**, as indicated by blue)(left middle panels) as well as

microbeads that associated with the cells (**Outside** Cy3–positive beads, as indicated by red)(right panels) as described in Materials and Methods. The outside or internalized microbeads were identified as Cy3/YG double-positive beads (arrows) or YG single-positive beads (arrowheads), respectively. Differential interference contrast (**DIC**) images were also acquired to determine cell shapes (left panels). Bar, 20 μm. (**B**) HEK293A cells were transfected and treated as in (A). The numbers of total YG–positive microbeads (**Total**) or YG–positive but Cy3–negative microbeads (**Internalized**) per a single CC20-expressing cell were counted (left panel) and thereafter the percentage of internalized microbeads per total YG–positive microbeads was determined (right panel). Data are means ± SE from 20 cells for each condition and are representative of three independent experiments. ***$p < 0.001$ for the indicated comparisons. (Mann-Whitney *U* test).

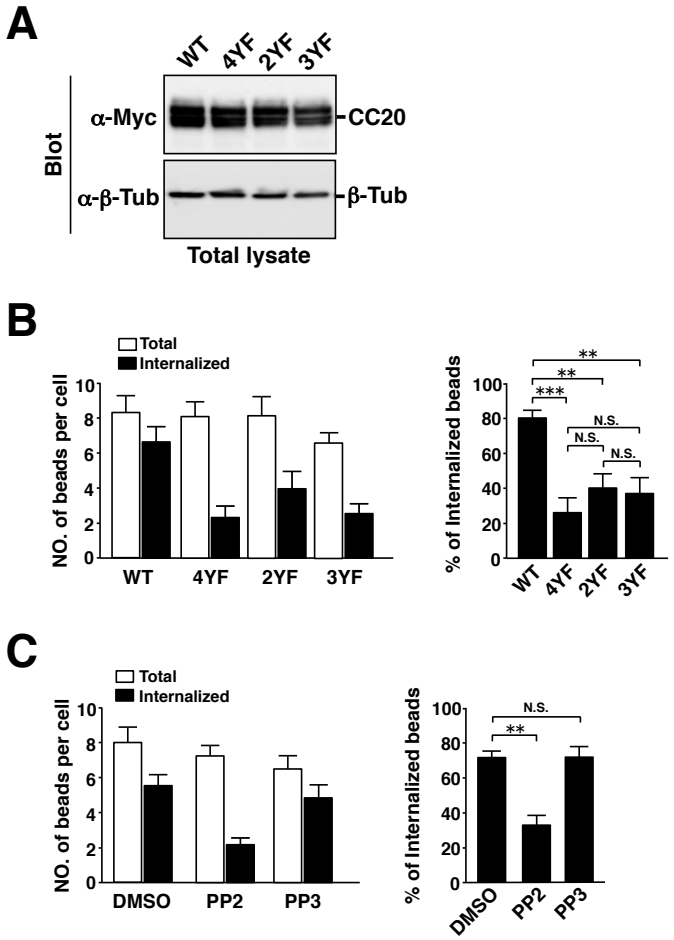

Figure 3. Importance of tyrosine phosphorylation of CEACAM20 for the promotion of phagocytic activity. (**A**) HEK293A cells were transfected with an expression vector for either MycCC20-WT, -4YF, -2YF or -3YF. Twelve h after transfection, cells were replated on cover glasses coated with poly-L-lysine and cultured for additional 12 h. Cells were then lysed and total cell lysates were subjected to immunoblot with mAbs to c-Myc or β-tubulin (β-Tub, loading control). Data are representative from three independent experiments. (**B**) HEK293A cells were transfected as in (A). Twelve h after transfection, the cells were subjected to the assay for phagocytic activity as in Fig. 2. (**C**) HEK293A cells that were

transfected with an expression vector for MycCC20-WT were pretreated with vehicle (dimethyl sulfoxide: DMSO), 5 μM PP2 or 5 μM PP3 for 30 min, after which they were subjected to the assay for phagocytic activity. The numbers of total YG–positive microbeads (Total) or YG–positive but Cy3–negative microbeads (Internalized) per a single CC20-expressing cell were counted (left panel) and thereafter the percentage of internalized microbeads per total YG–positive microbeads was determined (right panel). Data in (B) or (C) are means ± SE from 20 cells for each condition and are representative of three independent experiments. $**p < 0.01$, $***p < 0.001$ for the indicated comparisons. (Kruskal-Wallis test followed by Dunn's test). N.S., not significant.

Requirement of actin cytoskeleton reorganization, as well as activation of Syk, phosphoinositide 3-kinase (PI3K), or phospholipase C-γ (PLCγ), for the CEACAM20-mediated phagocytic activity

The reorganization of actin cytoskeleton is crucial for the promotion of phagocytosis [5]. Treatment of CEACAM20 WT-expressing cells with Cytochalasin D, an inhibitor for actin polymerization, significantly inhibited the internalization of anti-Myc mAb-coupled microbeads into the cells, whereas such treatment did not changed the total number of beads associated with CEACAM20 WT-expressing cells (**Fig. 4A**).

We further examined which signaling molecules are important for the CEACAM20-mediated phagocytic activity. Treatment with either Bay61-3606, an inhibitor for Syk [23], LY294002, an inhibitor for PI3K, or U73122, an inhibitor for PLCγ, markedly prevented the internalization of the anti-Myc mAb-coupled microbeads into CEACAM20 WT-expressing cells (**Fig. 4B**), suggesting that the activation of these signaling molecules is important for the CEACAM20-mediated phagocytic activity.

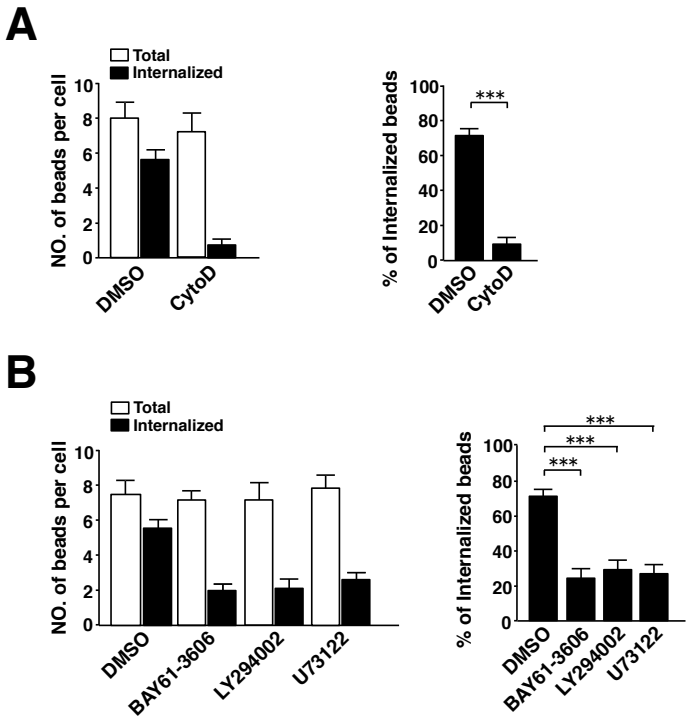

Figure 4. Requirement of actin cytoskeleton reorganization, as well as activation of Syk, PI3K or PLCγ, for the CEACAM20-mediated phagocytic activity. HEK293A cells were transfected with an

expression vector for MycCC20-WT. Twelve h after transfection, cells were replated on cover glasses coated with poly-L-lysine and cultured for additional 12 h. The cells were then pretreated with vehicle (**DMSO**) (**A, B**), 10 μM Cytochalasin D (**CytoD**) (**A**), 5 μM BAY61-3606 (**B**), 5 μM LY294002 (**B**) or 5 μM U73122 (**B**) for 30 min, after which they were incubated with anti-Myc mAb-coupled microbeads for 4 h and immunostained as in Fig. 2A. The numbers of total YG–positive microbeads (**Total**) or YG–positive but Cy3–negative microbeads (**Internalized**) per a single CC20-expressing cell were counted (left panel) and thereafter the percentage of internalized microbeads per total YG–positive microbeads was determined (right panel). Data are means ± SE from 20 cells for each condition and are representative of three independent experiments. ***$p < 0.001$ for the indicated comparisons. (Kruskal-Wallis test followed by Dunn's test).

DISCUSSION

We have here shown that tyrosine phosphorylation of CEACAM20 is promoted by treatment with PV or forced expression of c-Src. In addition, Tyr^{522}, Tyr^{559} or Tyr^{570}, the latter two of which are present at the ITAM in the cytoplasmic region of CEACAM20, is likely important for tyrosine phosphorylation of CEACAM20. We also demonstrated that forced expression of CEACAM20 promotes the phagocytic activity of cultured cells. Moreover, Tyr^{559} or Tyr^{570} at the ITAM is also important for the CEACAM20-mediated phagocytic activity. Consistently, inhibition by an SFK inhibitor, PP2, prevents the CEACAM20-mediated phagocytic activity. Thus, phosphorylation by SFKs of Tyr^{559} or Tyr^{570} at the ITAM is likely important for the CEACAM20-mediated phagocytic activity. The engagement of FcγR with IgG-opsonized particles on cell surface is thought to promote the phosphorylation by SFKs of the ITAM in the cytoplasmic region of FcγR [6,17]. Such phosphorylation of FcγR ITAM then promotes the binding of Syk through its SH2 domains [6,17]. The surrounding sequences of either Tyr^{559} or Tyr^{570} (namely Y^{559}EKL and Y^{570}CKI), at the ITAM of CEACAM20 correspond to those previously illustrated as a binding site for the SH2 domains of Syk, SFKs, Fps/Fes and Shc [20,21]. We indeed showed that Bay61-3606, an inhibitor for Syk, also prevents the CEACAM20-mediated phagocytic activity. Taken together, Syk or SFK is a potential binding partner for the ITAM of CEACAM20 and these signaling molecules are likely important for the CEACAM20-mediated phagocytic activity. We also showed that an inhibitor for either PI3K or PLCγ prevents the CEACAM20-mediated phagocytic activity. PI3K or PLCγ also contains the SH2 domains, the surrounding sequences of Tyr^{559} or Tyr^{570} at the ITAM of CEACAM20 do not match to the potential binding sites for the SH2 domains of these two signaling molecules [20], however. Thus, PI3K or PLCγ is unlikely a binding partner for the ITAM of CEACAM20, but they might play important roles for the signaling pathway downstream of CEACAM20.

In contrast to the roles of the cytoplasmic region of CEACAM20, the physiological function of the extracellular region of this protein remains unclear. CEACAM1, -3, -5 and -6 are thought to interact with bacteria, such as *Neisseria gonorrhoeae* (for CEACAM1, -3, -5 and -6), *Escherichia coli* (for CEACAM1, -5 and -6), *Salmonella typhi* (for CEACAM5 and -6) and *Haemophilus influenza* (for CEACAM1), through their extracellular domains [7]. In particular, interaction of the N-domain of CEACAM3 with Opa_{CEA}-expressing gonococci promotes tyrosine phosphorylation by SFKs of CEACAM3 ITAM-like motif and it promotes phagocytosis by granulocytes of the pathogens [4]. Of interest is that CEACAM20 mRNA is specifically expressed in the intestine [24]. Although it remains unknown whether CEACAM20 is indeed expressed in the intestinal epithelial cells, it is thus possible that CEACAM20 might interact with commensal or pathogenic microbes in the intestine,

resulting in its tyrosine phosphorylation and promotion of phagocytosis of the microbes. Alternatively, CEACAM20 might interact with other CEACAM family members by their extracellular regions. Indeed, CEACAM1 or CEACAM6 participates in intercellular adhesion by interacting with CEACAM5 through their extracellular domains [7]. Given that CEACAM1, -5 and -6 are expressed in intestinal epithelium [7], CEACAM20 might also interact with these CEACAMs *in trans* through their extracellular domains.

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