Protein tyrosine phosphatase SAP-1 protects against colitis through regulation of CEACAM20 in the intestinal epithelium

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Intestinal epithelial cells contribute to regulation of intestinal immunity in mammals, but the detailed molecular mechanisms of such regulation have remained largely unknown. Stomach-cancer–associated protein tyrosine phosphatase 1 (SAP-1, also known as PTPRH) is a receptor-type protein tyrosine phosphatase that is localized specifically at microvilli of the brush border in gastrointestinal epithelial cells. Here we show that SAP-1 ablation in interleukin (IL)-10–deficient mice, a model of inflammatory bowel disease, resulted in a marked increase in the severity of colitis in association with up-regulation of mRNAs for various cytokines and chemokines in the colon. Tyrosine phosphorylation of carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 20, an intestinal microvillus-specific transmembrane protein of the Ig superfamily, was greatly increased in the intestinal epithelium of the SAP-1–deficient animals, suggesting that this protein is a substrate for SAP-1. Tyrosine phosphorylation of CEACAM20 by the protein tyrosine kinase c-Src and the consequent association of CEACAM20 with spleen tyrosine kinase (Syk) promoted the production of IL-8 in cultured cells through the activation of nuclear factor-κB (NF-κB). In addition, SAP-1 and CEACAM20 were found to form a complex through interaction of their ectodomains. SAP-1 and CEACAM20 thus constitute a regulatory system through which the intestinal epithelium contributes to intestinal immunity.

colitis | intestinal epithelial cells | intestinal immunity | protein tyrosine phosphatase

Intestinal epithelial cells (IECs) play a central role in food digestion and absorption of nutrients, water, and electrolytes. They also contribute to the regulation of intestinal immunity by subserving two main functions (1). First, the single layer of IECs provides a physical barrier that protects the lamina propria as well as the inner body from the external environment, which includes the vast array of microbes present in the intestinal lumen. This barrier function of IECs is achieved through intercellular adhesion mediated by tight junctions, adherens junctions, and desmosomes (2). Indeed, mice deficient in the tight-junction component JAM-A manifest increased paracellular permeability and inflammation in the intestine (3). Moreover, forced expression of a dominant negative mutant of N-cadherin, which attenuated expression of endogenous E-cadherin, a major component of adherens junctions, also resulted in colonic inflammation in mice (4). The importance of the epithelial barrier for intestinal immunity is further supported by the finding of abnormal intestinal permeability in first-degree relatives of individuals with inflammatory bowel disease (IBD) such as Crohn's disease (5).

The second function of IECs related to regulation of intestinal immunity is the production of a variety of antimicrobial peptides—such as α- and β-defensin, which are produced by Paneth cells and prevent the growth of pathogenic microbes (6)—as well as of mucus, which is produced mostly by goblet cells. Indeed, a reduced level of α-defensin in the intestine is frequently associated with Crohn's disease (7). In addition, homozygous mutations of nucleotide oligomerization domain protein 2 (Nod2), an intracellular receptor for muramyl dipeptide, are highly associated with the incidence of Crohn's disease (8), with Nod2 also having been found to promote expression of the defensin-related cryptdins (9). The importance of Paneth cells for regulation of intestinal immunity was further revealed by the observation that ablation of the transcription factor XBP1 in IECs resulted in a loss of Paneth cells as well as development of enteritis in mice (10). Mucin2 (Muc2) is the most abundant mucin of intestinal mucus, and ablation of Muc2 in mice was found to result in the spontaneous development of colitis (11, 12). In contrast to such a role for IECs in protection against colitis, IECs are thought to contribute to the development of inflammatory infiltrates by producing chemokines, such as interleukin (IL)-8 in humans or its homologs keratinocyte-derived chemokine (KC) and macrophage inflammatory protein 2 (MIP-2) in mice (13, 14).

Significance

Much attention has been recently paid to the role of intestinal epithelial cells in the homeostatic regulation of intestinal immunity. Here we show that ablation of stomach-cancer–associated protein tyrosine phosphatase 1 (SAP-1) markedly increased the severity of colitis in interleukin (IL)-10–deficient mice, suggesting that SAP-1 protects against colitis in a cooperative manner with IL-10. We also identify carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 20, an intestinal microvilli-specific membrane protein, as a dephosphorylation target for SAP-1. Indeed, tyrosine phosphorylation of CEACAM20 promotes the binding of spleen tyrosine kinase (Syk) and activation of nuclear factor-κB (NF-κB), thereby inducing production of chemokines such as IL-8. Thus, we propose a mechanism by SAP-1 and CEACAM20 in the intestinal epithelium for regulation of the intestinal immunity.

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Moreover, proper turnover of IECs through regulation of cell death was shown to be important for homeostasis of intestinal immunity (15, 16). However, the detailed molecular mechanisms by which IECs regulate intestinal immunity have remained poorly elucidated.

Stomach-cancer–associated protein tyrosine phosphatase 1 (SAP-1, also known as PTPRH) is a receptor-type protein tyrosine phosphatase (PTP) with a single catalytic domain in its cytoplasmic region and multiple fibronectin type III-like domains in its extracellular region (17). It was previously shown to be localized specifically to microvilli of the brush border in epithelial cells of the small intestine and stomach in mice (18). SAP-1–deficient mice manifest no marked changes in the morphology of the small intestinal epithelium (18), suggesting that SAP-1 is not important for determination of the cellular architecture of this tissue. Moreover, SAP-1 is dispensable for regulation of food digestion and absorption of nutrients and electrolytes in the intestine. In contrast, forced expression of SAP-1 in cultured cells was shown to inhibit cell proliferation, an effect mediated in part by attenuation of growth-factor–induced mitogen-activated protein kinase (MAPK) activation or by induction of caspase-dependent apoptosis (19, 20). We have now investigated the potential role of SAP-1 in the regulation of intestinal immunity by IECs.

Results

Impact of SAP-1 Ablation on Development of Colitis in IL-10–Deficient Mice. SAP-1 was previously shown to be localized specifically to microvilli of the brush border in epithelial cells of the small intestine and stomach of mice (18). Immunohistofluorescence analysis showed that SAP-1 is also localized at the apical surface of colonic epithelial cells in the mouse (Fig. 1*A*). In addition, immunoelectron microscopy revealed prominent SAP-1 staining at the microvilli of epithelial cells in the colon (Fig. 1*B*). In contrast, SAP-1 immunoreactivity was virtually undetectable in the colon of SAP-1–deficient ($Sap1^{-/-}$) mice (Fig. 1 *A* and *B*), indicating that SAP-1 is indeed expressed in the microvilli of colonic epithelial cells. Not only intestinal immune cells but also IECs are thought to contribute to intestinal immunity (1). However, $Sap1^{-/-}$ mice up to 20 wk of age did not exhibit any sign of colonic inflammation, as judged on the basis of both clinical manifestations such as bloody stool or weight loss as well as histological examination (Fig. S1 *A* and *B*). We therefore crossed $Sap1^{-/-}$ mice with IL-10–deficient ($Il10^{-/-}$) mice, a model of human IBD such as Crohn's disease or ulcerative colitis (21, 22) and examined the impact of SAP-1 ablation on the severity of spontaneous colitis in these latter mice. We first monitored disease activity, which was scored on the basis of stool consistency, blood in the stool, and anorectal prolapse, in $Il10^{-/-}$ and $Il10^{-/-}Sap1^{-/-}$ mice at 10, 15, and 20 wk of age. Disease activity in $Il10^{-/-}Sap1^{-/-}$ mice (male and female) at 10–20 wk of age was markedly increased compared with that in $Il10^{-/-}$ mice (Fig. 1*C*). In particular, the incidence of anorectal prolapse was greatly increased in $Il10^{-/-}Sap1^{-/-}$ mice compared with $Il10^{-/-}$ mice (Fig. 1*D*). The colon of $Il10^{-/-}Sap1^{-/-}$ mice at 20 wk of age showed signs of severe colitis, including pronounced thickening of the bowel wall, a shortened colonic length, and unformed or absent stools, compared with $Il10^{-/-}$, $Sap1^{-/-}$, or wild type (WT) mice (Fig. S1 *C* and *D*). Microscopic examination revealed that the thickness of the colonic mucosa in $Il10^{-/-}Sap1^{-/-}$ mice at 20 wk of age was markedly increased compared with that in $Il10^{-/-}$ mice (Fig. 1*E*). In addition, severe epithelial hyperplasia, crypt distortion, crypt abscesses, and microadenoma were apparent in the colon of $Il10^{-/-}Sap1^{-/-}$ mice (Fig. S2). The histological score for colonic inflammation was thus significantly greater for $Il10^{-/-}Sap1^{-/-}$ mice than for $Il10^{-/-}$ mice (Fig. 1*F*). Finally, the survival rate of $Il10^{-/-}Sap1^{-/-}$ mice was substantially reduced compared with that of $Il10^{-/-}$ mice (Fig. 1*G*). Collectively, these observations thus suggested that SAP-1 ablation results in exacerbation of spontaneous colitis in $Il10^{-/-}$ mice.

Consistent with the extent of colonic inflammation, quantitative reverse transcription (RT)-PCR analysis revealed that the amounts

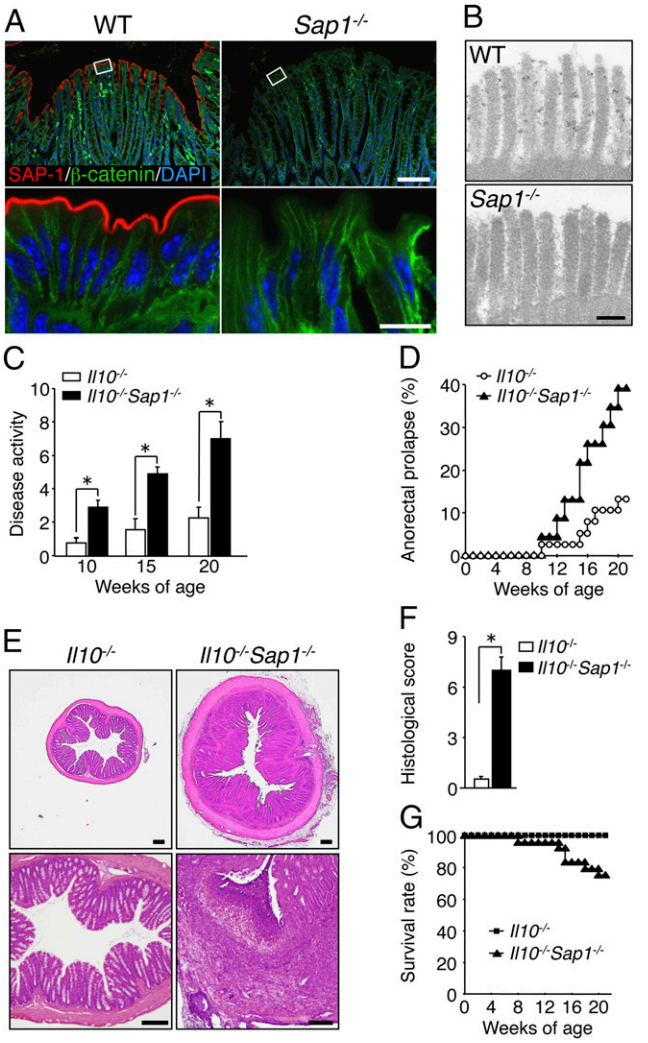

Fig. 1. Impact of SAP-1 ablation on development of colitis in IL-10–deficient mice. (*A*) Cryostat sections of the colon of WT or $Sap1^{-/-}$ mice at 6 wk of age were subjected to immunofluorescence analysis with antibodies to SAP-1 (red) and to β-catenin (green). Nuclei were also stained with DAPI (blue). Boxed regions (*Upper*) are shown at higher magnification (*Lower*). [Scale bars, 100 μm (*Upper*) or 10 μm (*Lower*).] (*B*) Immunoelectron microscopy of the colonic epithelium of adult WT or $Sap1^{-/-}$ mice with antibodies to SAP-1. (Scale bar, 200 nm.) (*C*) Disease activity for colitis in 10-, 15-, or 20-wk-old $Il10^{-/-}$ ($n = 35$, 18, and 19, respectively) or $Il10^{-/-}Sap1^{-/-}$ ($n = 40$, 24, and 24, respectively) mice. $^*P < 0.05$ (Mann–Whitney *u* test). (*D*) Incidence of anorectal prolapse in $Il10^{-/-}$ ($n = 38$) or $Il10^{-/-}Sap1^{-/-}$ ($n = 23$) mice. (*E*) H&E staining of midcolon sections from 20-wk-old $Il10^{-/-}$ or $Il10^{-/-}Sap1^{-/-}$ mice. (Scale bars, 200 μm.) (*F*) Histological score for inflammation in the colon of 20-wk-old $Il10^{-/-}$ or $Il10^{-/-}Sap1^{-/-}$ mice. $^*P < 0.05$ (Mann–Whitney *u* test). (*G*) Survival rates of $Il10^{-/-}$ ($n = 36$) and $Il10^{-/-}Sap1^{-/-}$ ($n = 24$) mice. Data are representative of three independent experiments (*A*, *B*, and *E*) and pooled from at least three independent experiments (*C*, *D*, and *G*); means ± SEM in *C*, or are from one representative experiment (*F*; means ± SEM for a total of five mice for each genotype).

of mRNAs for proinflammatory cytokines including tumor necrosis factor α (TNF-α), IL-6, IL-1β, and IL-12 were markedly increased in the colonic mucosa of $Il10^{-/-}Sap1^{-/-}$ mice at 10 wk of age compared with those in $Il10^{-/-}$, $Sap1^{-/-}$, or WT mice (Fig. 2*A*). The abundance of mRNAs for interferon γ (IFN-γ) and IL-17, both of which are implicated in development of colitis in IL-10–deficient mice (22, 23), was also increased in the colonic mucosa of $Il10^{-/-}Sap1^{-/-}$ mice (Fig. 2*A*). Conversely, the amounts of mRNAs for the T helper 2 cytokines IL-4 and IL-13 were decreased in the double-mutant animals. The expression of chemokine genes such as

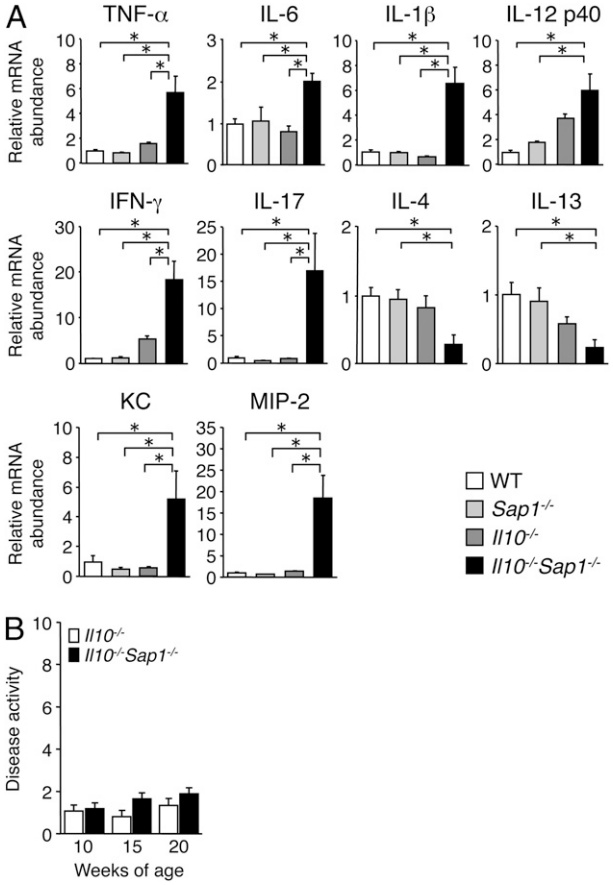

Fig. 2. Altered cytokine and chemokine mRNA abundance in the colon of *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice as well as the effect of antibiotic treatment on colitis development. (*A*) Quantitative RT-PCR analysis of cytokine and chemokine mRNAs in the colon of 10-wk-old WT, *Sap1*$^{-/-}$, *Il10*$^{-/-}$, or *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice. The amount of each mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) mRNA and then expressed relative to the normalized value for WT mice. *$P < 0.05$ (ANOVA and Tukey's test). (*B*) *Il10*$^{-/-}$ ($n = 15$) and *Il10*$^{-/-}$*Sap1*$^{-/-}$ ($n = 17$) mice were treated with antibiotics from 4 wk of age, and disease activity for colitis was determined at 10, 15, and 20 wk of age. Data are from one representative experiment (*A*; means ± SEM for a total of five mice for each genotype) or are pooled from at least three independent experiments (*B*; means ± SEM).

those for KC and MIP-2 in the colonic mucosa was greatly up-regulated in *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice compared with the other three strains (Fig. 2*A*). These results thus further suggested that SAP-1, together with IL-10, protects against the development of colitis. Commensal bacteria are implicated in the development of colitis in IL-10–deficient mice (21, 24). We therefore next examined the role of commensal bacteria in the exacerbation of colitis in *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice. Mice were given a combination of broad-spectrum antibiotics in drinking water beginning at 4 wk of age to deplete commensal bacteria. Such depletion largely prevented the development of colitis in both *Il10*$^{-/-}$*Sap1*$^{-/-}$ and *Il10*$^{-/-}$ mice at 10–20 wk of age, with scores for disease activity in the two strains being similar (Fig. 2*B*). These results suggested that commensal bacteria are important for the increase in the severity of spontaneous colitis induced by SAP-1 ablation in *Il10*$^{-/-}$ mice.

Identification of Carcinoembryonic Antigen-Related Cell Adhesion Molecule (CEACAM) 20 as a Tyrosine-Phosphorylated Protein in the Intestinal Epithelium of SAP-1–Deficient Mice. We further investigated the molecular mechanism by which ablation of SAP-1 exacerbates colitis in *Il10*$^{-/-}$ mice. SAP-1 was previously shown to inhibit the proliferation of cultured cells (19, 20). However, cell turnover, morphology of intercellular junctions, and paracellular permeability in the colonic epithelium did not differ between WT and *Sap1*$^{-/-}$ mice (Fig. S3). In addition, the number of goblet cells or Paneth cells, which are thought to protect against colitis by secreting mucus or antimicrobial peptides, respectively, in the intestinal epithelium did not differ between the two strains (Fig. S4). In contrast, immunohistofluorescence analysis with antibodies to phosphotyrosine revealed that staining was markedly increased along the apical surface of colonic epithelial cells in *Sap1*$^{-/-}$ mice compared with WT mice (Fig. 3*A*). Consistent with this observation, immunoblot analysis of isolated microvillus membranes from the small intestine with the same antibodies showed that the levels of tyrosine phosphorylation

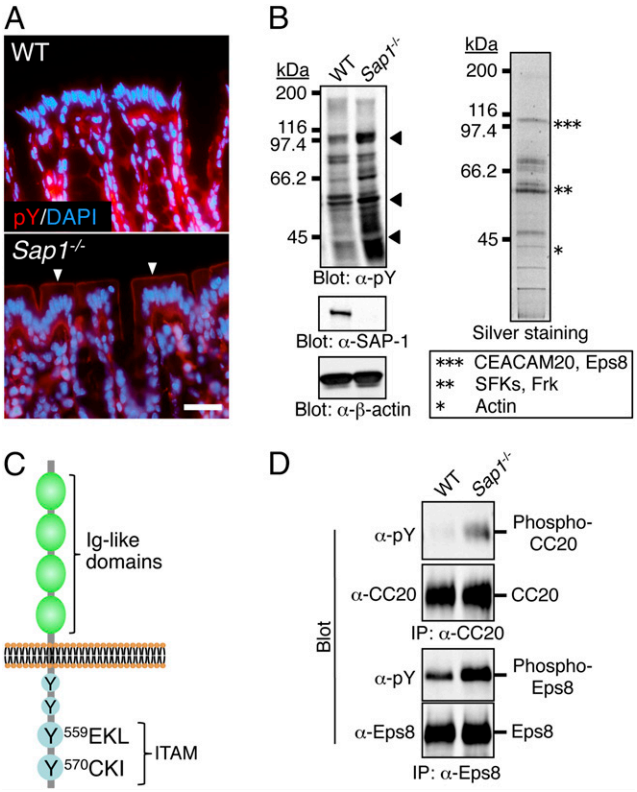

Fig. 3. Identification of CEACAM20 as a tyrosine-phosphorylated protein in the intestinal epithelium of SAP-1–deficient mice. (*A*) Sections of the colon of 10-wk-old WT or *Sap1*$^{-/-}$ mice were stained with antibodies to phosphotyrosine (pY, red) and with DAPI (blue). (Scale bar, 20 μm.) Arrowheads indicate prominent staining for phosphotyrosine along the apical surface of the colonic epithelium in the mutant. (*B*) Microvillus membranes prepared from the entire small intestine of WT or *Sap1*$^{-/-}$ mice were subjected to immunoblot analysis with antibodies to phosphotyrosine (α-pY), to SAP-1, or to β-actin (*Left*). Bands corresponding to proteins whose level of tyrosine phosphorylation was markedly increased in *Sap1*$^{-/-}$ mice are indicated by arrowheads. Tyrosine-phosphorylated proteins purified from a solubilized microvillus membrane fraction of *Sap1*$^{-/-}$ mice with the use of agarose-bead–conjugated antibodies to phosphotyrosine were fractionated by SDS/PAGE and visualized by silver staining (*Right*). The protein bands indicated by the asterisks were analyzed by MS. The ~100-, ~60-, and ~40-kDa protein bands (***, **, and *) contained the indicated proteins. (*C*) Schematic representation of the structure of mouse CEACAM20 showing four Ig-like domains in the extracellular region and four potential tyrosine phosphorylation sites, two of which constitute an ITAM, in the cytoplasmic region. (*D*) Microvillus membranes prepared from the entire small intestine of WT or *Sap1*$^{-/-}$ mice were subjected to immunoprecipitation (IP) with antibodies to CEACAM20 (α-CC20) or to Eps8, and the resulting precipitates were subjected to immunoblot analysis of phosphotyrosine, CEACAM20, or Eps8. Data are representative of three (*A*) or two (*B* and *D*) independent experiments.

of several proteins (molecular sizes of ~40 to ~110 kDa) were increased in *Sap1*$^{-/-}$ mice (Fig. 3*B*). Tyrosine-phosphorylated proteins were affinity purified from the solubilized microvillus membrane fraction of *Sap1*$^{-/-}$ mice with the use of agarose beads conjugated with the antibodies to phosphotyrosine and were then visualized by silver staining of SDS/PAGE gels (Fig. 3*B*). Bands corresponding to ~100-, ~60-, and ~40-kDa proteins were excised, enzymatically digested, and subjected to mass spectrometry (MS). Several peptide fractions were obtained for each protein band, and the molecular size of these peptides was determined by MALDI-TOF MS. Comparison of the determined molecular sizes with theoretical peptide masses for proteins registered in the nonredundant database in the National Center for Biotechnology Information (NCBInr) indicated that the ~100-kDa protein band contained CEACAM20 and epidermal growth factor receptor kinase substrate 8 (Eps8) (Fig. 3*B*). In addition, the ~60-kDa protein band contained several Src family kinase SFKs (Lyn, c-Yes, Lck, c-Src, and c-Fgr) as well as the SFK-related protein Frk (Fig. 3*B*).

On the basis of its cDNA sequence, CEACAM20 is predicted to be a transmembrane protein that possesses four Ig-like domains in its extracellular region as well as four potential tyrosine phosphorylation sites in its cytoplasmic region, with the two COOH-terminal tyrosine residues (Tyr^{559} and Tyr^{570}) and their surrounding sequence corresponding well to the immunoreceptor tyrosine-based activation motif (ITAM) (25) (Fig. 3*C*). Immunoprecipitation with antibodies to mouse CEACAM20 showed that the extent of tyrosine phosphorylation of this protein was indeed increased in the microvillus membrane fraction of *Sap1*$^{-/-}$ mice compared with that apparent for WT mice (Fig. 3*D*). These results thus suggested that CEACAM20 is a substrate for the PTP activity of SAP-1 in the intestinal epithelium. In contrast, the phosphorylation of Tyr^{416} of c-Src (26) as well as the tyrosine phosphorylation of other SFKs in microvillus membranes did not differ substantially between *Sap1*$^{-/-}$ and WT mice (Fig. S5). The extent of tyrosine phosphorylation of Eps8 was also increased in microvillus membranes of *Sap1*$^{-/-}$ mice compared with that apparent for WT mice (Fig. 3*D*). However, given that the abundance of *Ceacam20* mRNA, like that of *Sap1* mRNA, was previously found to be highest in the intestine (18, 25), whereas Eps8 is expressed ubiquitously (27), we pursued the further characterization of CEACAM20 as a potential substrate for SAP-1 in the intestinal epithelium.

Colocalization of CEACAM20 and SAP-1 in the Intestinal Epithelium. We next examined the localization and function of CEACAM20. Immunoblot analysis of various mouse tissues showed that the abundance of CEACAM20 was highest in the small intestine and colon, being minimal or low in other tissues (Fig. 4*A*). This expression pattern of CEACAM20 is essentially identical to that of SAP-1 (Fig. 4*A*) (18). Immunohistofluorescence analysis revealed that staining for CEACAM20 was localized at the apical surface of the colon and largely overlapped with that of SAP-1 (Fig. 4*B*). Immunoreactivity for CEACAM20 was detected immediately above prominent staining for F-actin, likely corresponding to the terminal web (2), at the brush border of colonic epithelial cells (Fig. 4*B*). These results thus indicated that CEACAM20 is expressed specifically in microvilli of colonic epithelial cells, where it colocalizes with SAP-1.

Coexpression of SAP-1 and Myc epitope-tagged CEACAM20 in HEK293A cells also revealed that the two proteins coimmunoprecipitated with each other (Fig. 4*C*). Such complex formation was also apparent when mutant versions of either or both proteins that lack the cytoplasmic region were coexpressed (Fig. 4*C*). By contrast, the association of SAP-1 with CEACAM1,

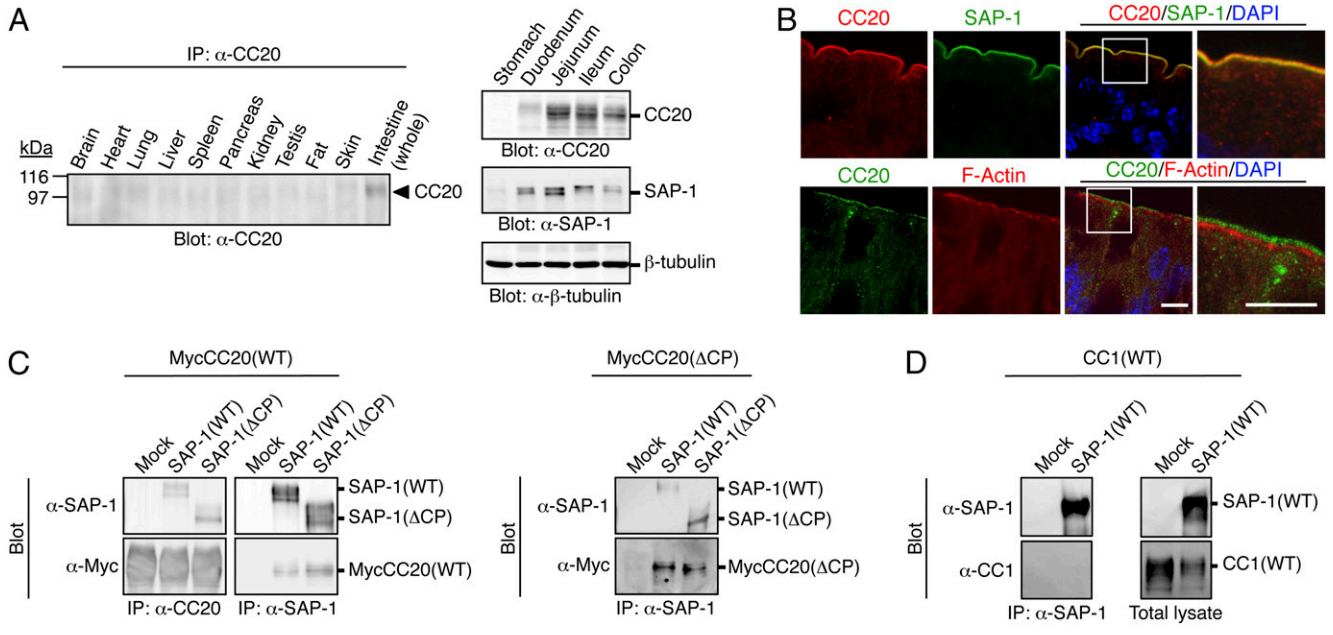

Fig. 4. Colocalization of CEACAM20 and SAP-1 in the intestinal epithelium. (*A*) Lysates of the indicated adult WT mouse tissues were subjected to immunoprecipitation with antibodies to CEACAM20 (α-CC20), and the resulting precipitates were subjected to immunoblot analysis with the same antibodies (*Left*). Lysates of mouse stomach, duodenum, jejunum, ileum, and colon were also subjected to immunoblot analysis of CEACAM20, SAP-1, or β-tubulin (*Right*). (*B*) Sections of the colonic epithelium of 8-wk-old WT mice were stained with antibodies to CEACAM20 and to SAP-1, with rhodamine-conjugated phalloidin for detection of F-actin, and with DAPI, as indicated. Boxed regions (*Middle Right*) are shown at higher magnification at *Right*. (Scale bars, 10 μm.) (*C*) HEK293A cells transfected with expression vectors for SAP-1(WT) and Myc-epitope–tagged CEACAM20(WT) [MycCC20(WT)], or for mutant versions of each protein lacking the cytoplasmic region (ΔCP), as indicated, were lysed in a cell lysis buffer containing *n*-octyl-β-D-glucoside (ODG buffer) as described in the *SI Materials and Methods* section and subjected to immunoprecipitation and immunoblot analysis with the indicated antibodies. (*D*) HEK293A cells transfected with expression vectors for SAP-1(WT) and CEACAM1(WT) [CC1(WT)] were lysed in ODG buffer and subjected to immunoprecipitation and immunoblot analysis with the indicated antibodies. Total cell lysates were also subjected directly to immunoblot analysis. All data are representative of three independent experiments.

another CEACAM family member expressed in colonic epithelial cells (28), was not detected in HEK293A cells overexpressing these two proteins (Fig. 4*D*). These results suggested that SAP-1 specifically interacts with CEACAM20 and that this interaction is mediated via the ectodomains of both proteins.

Tyrosine Phosphorylation of CEACAM20 by SFKs and Its Association with Spleen Tyrosine Kinase (Syk). Given that CEACAM20 was identified as a potential substrate for SAP-1 and was found to colocalize with SAP-1 in colonic epithelial cells, we further investigated the properties of this protein. Consistent with the presence of putative tyrosine phosphorylation sites in its cytoplasmic region (Fig. 3*C*), we found that forced expression of CEACAM20 tagged with the Myc epitope in HEK293A cells resulted in tyrosine phosphorylation of the overexpressed protein and that such phosphorylation was prevented by treatment of the cells with PP2, an inhibitor of SFKs, but not by that with PP3, an inactive PP2 analog (Fig. 5*A*). In addition, coexpression of c-Src or an activated form of the tyrosine kinase Fyn together with CEACAM20 in HEK293A cells markedly enhanced the tyrosine phosphorylation of the latter protein (Fig. 5 *B* and *C*), suggesting that SFKs play a role in the tyrosine phosphorylation of CEACAM20. Expression of SAP-1, but not that of its catalytically inactive mutants SAP-1(C/S) or SAP-1(D/A), reduced the extent of tyrosine phosphorylation of CEACAM20 in transfected cells (Fig. 5*D*). Incubation of tyrosine-phosphorylated CEACAM20 in vitro with a glutathione S-transferase (GST) fusion protein containing the cytoplasmic domain of SAP-1 [GST–SAP-1(WT)], but not that with GST–SAP-1(C/S), resulted in its efficient dephosphorylation (Fig. S6), further suggesting that CEACAM20 is likely a substrate for SAP-1. The ITAM of CEACAM20 contains Y559EKL and Y570CKI sequences (Fig. 3*C*), which correspond well to sequences previously shown to serve when phosphorylated as a binding site for the SH2 domains of the tyrosine kinase Syk or SFKs (29, 30). Indeed, coexpression of CEACAM20 and Syk in HEK293A cells resulted in the association of the two proteins as well as in a marked increase in the tyrosine phosphorylation of Syk, whereas a mutant of CEACAM20 [CEACAM20(2YF)] in which Tyr^{559} and Tyr^{570} are replaced with Phe failed to form a complex with Syk (Fig. 5*E*). A GST fusion protein containing the two SH2 domains of Syk also bound to tyrosine-phosphorylated CEACAM20(WT) but not to CEACAM20(2YF) in vitro (Fig. S7*A*). In contrast, tyrosine-phosphorylated CEACAM20 failed to bind to c-Src or Fyn as well as to the SFK-related protein Frk (Fig. S7 *B*–*D*), the latter of which is highly expressed in the intestine (31). Furthermore, coexpression of Syk with CEACAM20(WT) [but not that with CEACAM20(2YF)] resulted in an increase in the level of tyrosine phosphorylation of the latter protein (Fig. 5*F*). These data thus suggested that tyrosine-phosphorylated CEACAM20 specifically binds to the SH2 domains

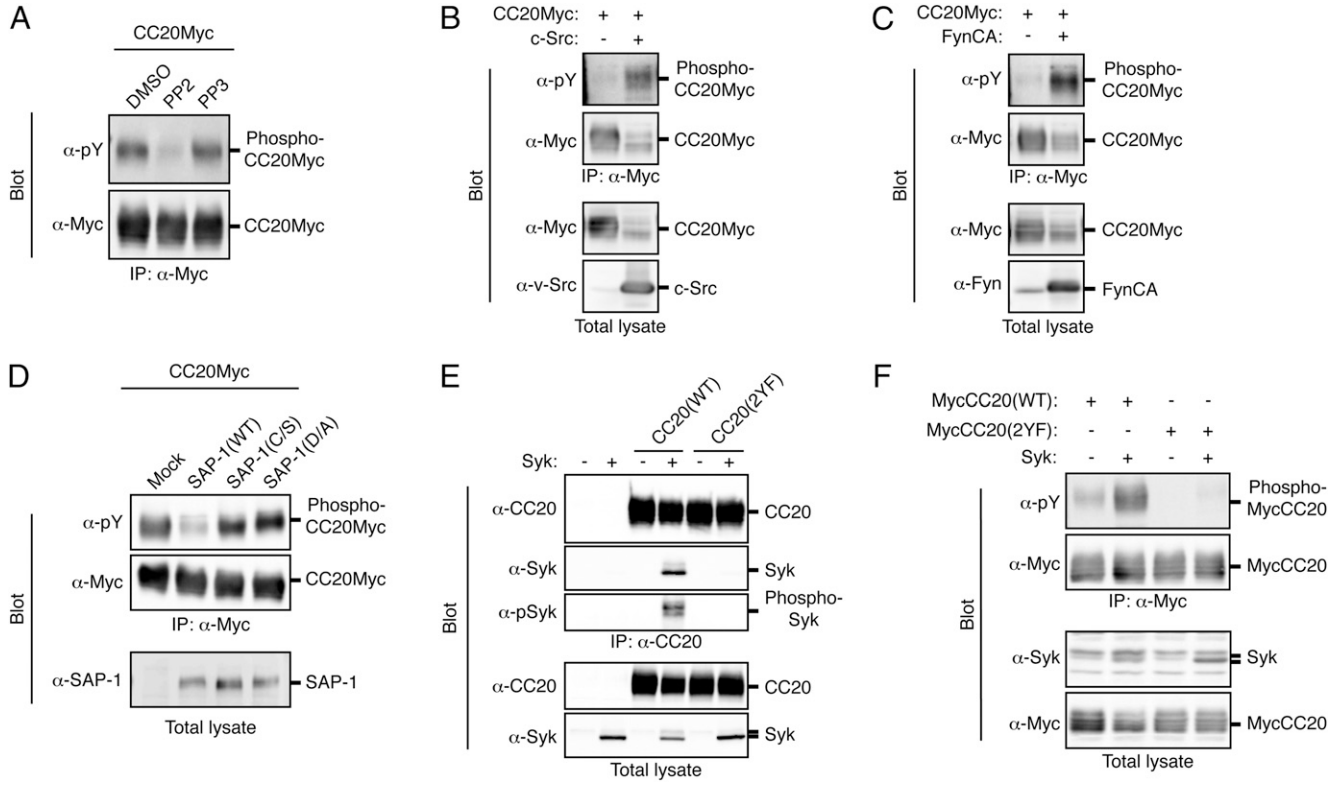

Fig. 5. Tyrosine phosphorylation of CEACAM20 by SFKs and its association with Syk. (*A*) HEK293A cells expressing Myc-epitope–tagged CEACAM20 (CC20Myc) were treated with 10 μM PP2 or PP3 (or with DMSO vehicle) for 30 min, after which cell lysates were subjected to immunoprecipitation with antibodies to Myc and the resulting precipitates were subjected to immunoblot analysis with antibodies to Myc or to phosphotyrosine (α-pY). (*B* and *C*) Lysates of HEK293A cells transfected with an expression vector for CC20Myc together with either an expression vector for c-Src (*B*), an active mutant of Fyn [Fyn CA] (*C*), or the corresponding empty vector (*B* and *C*) were subjected to immunoprecipitation and immunoblot analysis as in *A*. Total cell lysates were also subjected to immunoblot analysis with antibodies to Myc (*B* and *C*), to v-Src (*B*), or to Fyn (*C*). (*D*) Lysates of HEK293A cells transfected with expression vectors for the indicated proteins were subjected to immunoprecipitation and immunoblot analysis as in *A*. Total cell lysates were also subjected to immunoblot analysis with antibodies to SAP-1. (*E*) Lysates of HEK293A cells transfected with expression vectors for the indicated proteins were subjected to immunoprecipitation with antibodies to CEACAM20 (α-CC20), and the resulting precipitates as well as the original cell lysates were subjected to immunoblot analysis with antibodies to CEACAM20, to Syk, or to phosphorylated Syk (α-pSyk). (*F*) Lysates of HEK293A cells transfected with expression vectors for the indicated proteins were subjected to immunoprecipitation with antibodies to Myc, and the resulting precipitates as well as the original cell lysates were subjected to immunoblot analysis with the indicated antibodies. All data are representative of three independent experiments.

of Syk and thereby activates this kinase, which in turn mediates the further tyrosine phosphorylation of CEACAM20. Immunohistofluorescence analysis showed that Syk was indeed present in the cytoplasm of colonic epithelial cells (Fig. S8).

CEACAM20 Promotes Chemokine Production Through Activation of Nuclear Factor-κB (NF-κB). IECs contribute to the regulation of intestinal immunity by producing chemokines such as IL-8, KC, and MIP-2 that promote inflammatory infiltration, in particular that of neutrophils (13, 14). We therefore examined whether CEACAM20 together with c-Src and Syk might promote chemokine production. Forced expression of CEACAM20 with c-Src in HEK293A cells (which express endogenous Syk) (Fig. 5 *E* and *F*) resulted in a significant increase in IL-8 production compared with that observed in cells expressing either protein alone (Fig. 6*A*). This effect of CEACAM20 and c-Src was further enhanced by coexpression of Syk (Fig. 6*A*). By contrast, forced expression of CEACAM20(2YF) together with c-Src and Syk had no effect on IL-8 production (Fig. 6*A*). In addition, coexpression of SAP-1 markedly attenuated the increase in IL-8 production induced by expression of CEACAM20 and c-Src (Fig. 6*B*). These results suggested that tyrosine phosphorylation of CEACAM20 and its association with Syk in the presence of c-Src promote IL-8 production, whereas SAP-1 counteracts this effect.

Activation of NF-κB and MAPKs downstream of Syk is thought to be important for production of proinflammatory cytokines or chemokines including IL-8 (32, 33). Indeed, treatment of HEK293A cells with the NF-κB inhibitors JSH-23 or pyrrolidinedithiocarbamate (PDTC), or with PD98059, an inhibitor of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), markedly attenuated the increase in IL-8 production induced by CEACAM20 plus c-Src and Syk (Fig. 6*C*). In contrast, treatment with SB203580 or SP600125, inhibitors of the MAPKs p38 MAPK and c-Jun amino-terminal kinase (JNK), respectively, had only a small or no inhibitory effect on such IL-8 production (Fig. 6*C*). Consistent with these findings, forced expression of CEACAM20(WT) [but not that of CEACAM20(2YF)] together with c-Src and Syk increased the expression of a luciferase reporter gene placed under the transcriptional control of NF-κB response elements (Fig. 6*D*). These results suggested that activation of NF-κB is important for promotion of IL-8 production by CEACAM20.

Given that we found that tyrosine phosphorylation of CEACAM20 and its association with Syk likely promote IL-8 production in cultured cells, we examined whether the expression of KC or MIP-2 occurs in the colonic epithelial cells of *Il10*$^{-/-}$ or *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice before the apparent onset of colitis. The levels of mRNAs for KC and MIP-2 in colonic epithelial cells isolated from the double-mutant mice at 6–8 wk of age (when the disease activity score was 0–2) were about four and six times, respectively, those in cells isolated from *Il10*$^{-/-}$ mice (Fig. 6*E*). In contrast, the amount of mRNA for TNF-α in the cells from *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice was increased only twofold relative to that for *Il10*$^{-/-}$ mice, whereas the abundance of mRNA for IL-6 did not differ substantially between the two strains (Fig. 6*E*). These results thus suggested that the expression of MIP-2 and KC tends to be increased in the intestinal epithelium of *Il10*$^{-/-}$ *Sap1*$^{-/-}$ mice before the development of colitis.

Discussion

We have here shown that ablation of SAP-1, a microvillus-specific PTP, exacerbated spontaneous colitis in association with upregulation of mRNAs for various cytokines and chemokines in the colon of *Il10*$^{-/-}$ mice, whereas *Sap1*$^{-/-}$ mice did not manifest

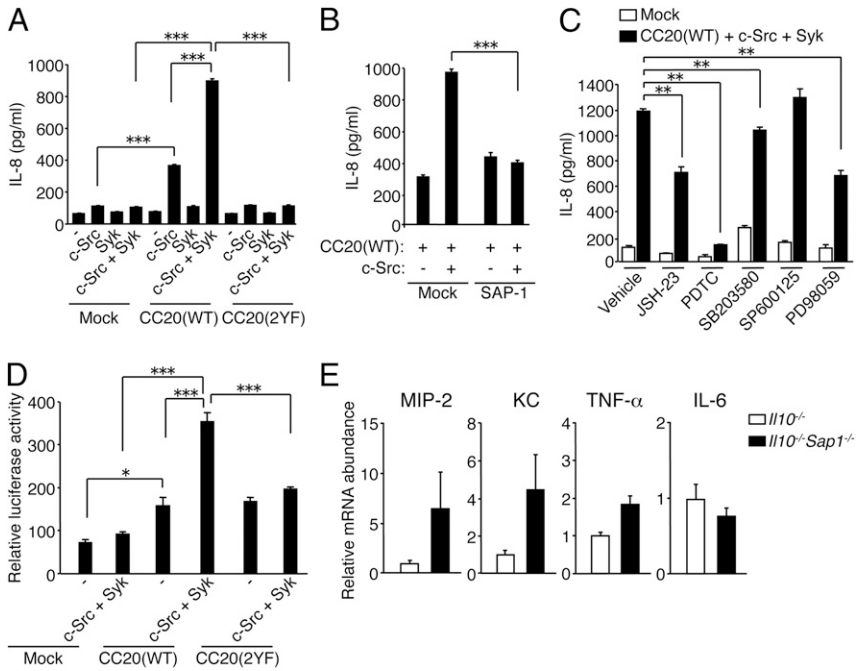

Fig. 6. CEACAM20 promotes chemokine production through activation of NF-κB. (*A*–*C*) HEK293A cells transfected with expression vectors for the indicated proteins were cultured for 24 h in the absence (*A* and *B*) or presence (*C*) of either DMSO (vehicle), NF-κB inhibitors (JSH-23 or PDTC), a p38 MAPK inhibitor (SB203580), a JNK inhibitor (SP600125), or a MEK inhibitor (PD98059). The concentration of IL-8 in the culture supernatants was then determined. $^{**}P < 0.01$, $^{***}P < 0.001$ (ANOVA and Tukey's test). (*D*) HEK293A cells transfected with expression vectors for the indicated proteins together with an NF-κB reporter plasmid and internal control plasmid were lysed and assayed for luciferase activity. $^{*}P < 0.05$, $^{***}P < 0.001$ (ANOVA and Tukey's test). (*E*) Quantitative RT-PCR analysis of mRNAs for MIP-2, KC, TNF-α, and IL-6 in colonic epithelial cells isolated from 6- to 8-wk-old *Il10*$^{-/-}$ or *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice. The amount of each mRNA was normalized by that of *Gapdh* mRNA and then expressed relative to the normalized value for *Il10*$^{-/-}$ mice. Data are representative of three independent experiments (*A*–*D*; means ± SEM of triplicates for each condition) or are from one representative experiment (*E*; means ± SEM for a total of 10 mice for each genotype).

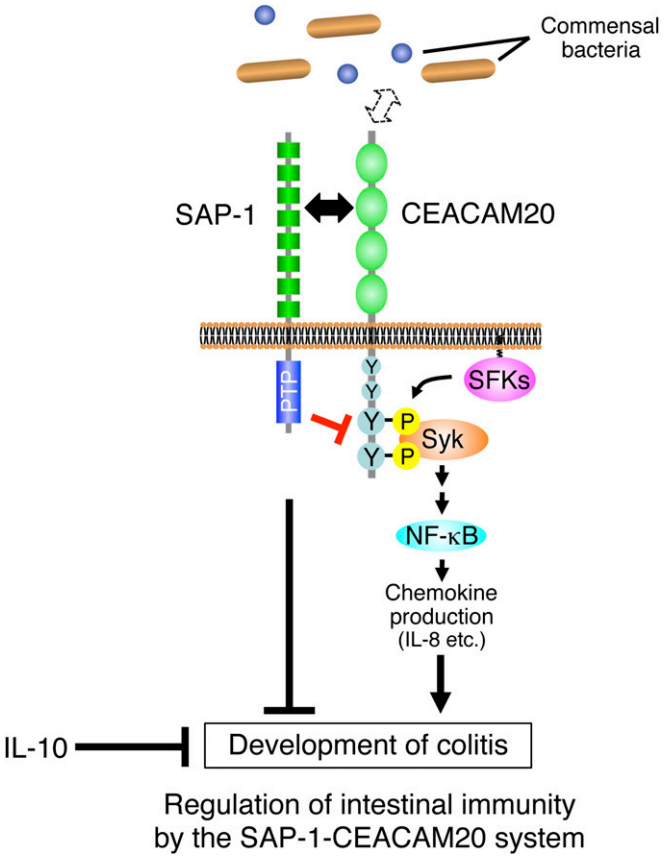

Fig. 7. Model for regulation of intestinal immunity by the SAP-1–CEACAM20 system. SAP-1 is a microvillus-specific PTP that together with IL-10 protects against the development of colitis. SAP-1 negatively regulates the function of CEACAM20 by mediating its dephosphorylation. CEACAM20 is also a microvillus-specific protein whose ectodomain likely interacts with that of SAP-1. It also possesses in its cytoplasmic region an ITAM, which is phosphorylated by SFKs and serves as a binding site for the SH2 domains of the tyrosine kinase Syk. The formation of a complex by tyrosine-phosphorylated CEACAM20 and Syk induces the activation of NF-κB and thereby increases the production of chemokines such as IL-8 and promotes inflammation of the intestinal mucosa.

any sign of colonic inflammation. IL-10 is thought to suppress the functions of various immune cells in the intestine, thereby protecting against colitis (34). Ablation of IL-10 in mice thus results in colonic inflammation that resembles IBD in humans (21, 22). Depletion of commensal bacteria with antibiotics also attenuates the severity of colitis in *Il10*$^{-/-}$ mice (21, 24), suggesting the importance of such bacteria in this colitis model. We found that antibiotic treatment also prevented the exacerbation of colitis induced by SAP-1 ablation in *Il10*$^{-/-}$ mice. Our results thus suggest that SAP-1, in cooperation with IL-10, contributes to protection against the development of colitis.

We also investigated the molecular mechanism by which SAP-1 regulates intestinal immunity and by which ablation of SAP-1 exacerbates colitis in *Il10*$^{-/-}$ mice. We found that the extent of tyrosine phosphorylation of CEACAM20, which is specifically expressed in IECs, was markedly increased in *Sap1*$^{-/-}$ mice. We also showed that tyrosine-phosphorylated CEACAM20 was efficiently dephosphorylated by SAP-1 in vitro as well as in cultured cells. Moreover, the expression pattern and localization of CEACAM20 overlapped with those of SAP-1, with both proteins being localized at microvilli of the intestine. Furthermore, SAP-1 and CEACAM20 were found to form a complex through interaction of their ectodomains in cultured cells, suggesting that both proteins physically associate with each other. Collectively, these observations suggest that CEACAM20 is a physiological substrate for SAP-1 in the intestinal epithelium.

Given that we found that SAP-1 regulates intestinal immunity through dephosphorylation of CEACAM20, we also investigated the function as well as signaling downstream of CEACAM20. We found that c-Src promotes the phosphorylation of Tyr^{559} or Tyr^{570} in the COOH-terminal region of CEACAM20 and that Syk then binds to tyrosine-phosphorylated CEACAM20 through its SH2 domains. Such binding to CEACAM20 likely results in the activation of Syk and promotes further tyrosine phosphorylation of CEACAM20. Finally, we showed that formation of the CEACAM20–Syk complex promoted the production of IL-8, likely as a result of the activation of NF-κB, in cultured cells. In contrast, forced expression of SAP-1 markedly attenuated the increase in IL-8 production induced by expression of CEACAM20 and c-Src, suggesting that SAP-1 counteracts the effect of tyrosine phosphorylation of CEACAM20 on IL-8 production. We have also found that forced expression of CEACAM20 together with c-Src and Syk induces the production of IL-6 in HEK293A cells (Fig. S9). CEACAM20 thus likely promotes inflammatory conditions in the intestine through its formation of a complex with Syk and the consequent production of chemokines and cytokines in IECs.

Neutrophil infiltration into the intestinal mucosa is fundamental to the development and progression of IBD (35). The chemokine IL-8 is thought to play a major role in the neutrophil infiltration that is frequently associated with colitis lesions in individuals with IBD (35). Mice deficient in chemokine (C-X-C motif) receptor 2 (CXCR2), a receptor for the IL-8 homologs MIP-2 and KC, manifest a reduced susceptibility to dextran sulfate sodium-induced colitis, another animal model of IBD (36). Conversely, transgenic mice that overexpress MIP-2 specifically in the intestinal epithelium manifest exaggeration of such colitis (14). The levels of mRNAs for MIP-2 and KC tended to increase in the intestinal epithelium of *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice before the development of colitis. The tyrosine-phosphorylation of CEACAM20 thus likely contributes at least in part to the development of colitis in *Il10*$^{-/-}$*Sap1*$^{-/-}$ mice.

We found that the ectodomain of SAP-1 interacts with that of CEACAM20. Given that both proteins are localized at microvilli of IECs, they—and in particular CEACAM20—might also interact with commensal bacteria in the intestine. Indeed, the Ig-like ectodomain of another CEACAM family member, CEACAM3, which also contains an ITAM-like motif in its cytoplasmic domain, recognizes bacteria that express the Opa protein and thereby triggers phagocytosis and elimination of the bacteria by granulocytes (37). In addition, CEACAM5 and CEACAM6, which are expressed in human IECs, have been shown to function as a receptor for *Escherichia coli* isolated from the intestine of healthy individuals or IBD patients (38, 39). Similarly, commensal or pathogenic bacteria might contribute to the regulation of CEACAM20 function by interacting with the ectodomain of this protein at the microvilli of IECs.

In summary, we propose a model for regulation of intestinal immunity by SAP-1 and CEACAM20 (Fig. 7). Further study will be required to elucidate whether mutations of the genes for SAP-1 or CEACAM20 are associated with IBD in humans. Nevertheless, these molecules are potential drug targets for the treatment of IBD.

Materials and Methods

Antibodies, reagents, mice, expression vectors, and detailed methods for gut commensal bacteria depletion, antibody generation, histological and immunofluorescence analyses, electron microscopy, clinical and histological assessment of colitis, mouse IEC isolation, RNA isolation and quantitative RT-PCR analysis, BrdU incorporation assay, in situ closed-loop system, microvillus membrane preparation, affinity purification and MS, cell culture and transfection, immunoprecipitation and immunoblot analysis, in vitro dephosphorylation assay, IL-6 and IL-8 production assay, reporter assay, and statistical analysis used in this study can be found in *SI Materials and Methods*. This study was approved by the Animal Care and Experimentation Committees of Kobe University and Gunma University.

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