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Membrane-anchored CD40 Is Processed by the Tumor Necrosis Factor-α-converting Enzyme

IMPLICATIONS FOR CD40 SIGNALING*

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Cécile Contin‡§, Vincent Pitard‡, Toshimitsu Itai¶, Shigekazu Nagata¶, Jean-François Moreau‡, and Julie Déchanet-Merville‡‖

From the ‡CNRS UMR 5540, IFR 66, Université Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux, France and the ¶Department of Genetics, Osaka University Medical School, Osaka 565-0871, Japan

The soluble form of CD40 (sCD40), which co-exists with the membrane-anchored form (mCD40), is a natural antagonist of mCD40/CD154 interaction. However, the mechanism leading to the production of sCD40 has never been investigated. Here, we show that the engagement of mCD40 on the surface of B lymphocytes by anti-CD40 antibody led to enhanced sCD40 release associated with decreased amounts of mCD40. This sCD40 production was not affected by vesicular traffic inhibitors but was completely blocked by a broad-spectrum synthetic metalloproteinase (MP) inhibitor (GM6001) or a membrane-anchored MP-specific inhibitor (dec-RVKR-cmk). Recombinant MP disintegrin tumor necrosis factor-α converting enzyme (TACE) cleaved the purified CD40 ectodomain/Fc chimeric protein *in vitro*, giving rise to an sCD40 form similar to that shed from B cell cultures. Moreover, spontaneous production of sCD40 by mCD40-transfected human embryonic kidney cells (constitutively expressing TACE) was enhanced by the overexpression of TACE and abrogated by co-transfection with a dominant-negative TACE mutant. These results provide strong evidence that sCD40 production is an active process regulated by the engagement of mCD40 and its proteolytic cleavage by TACE or a related MP disintegrin. Given the antagonistic activity of sCD40 on the CD40/CD154 interaction, this shedding mechanism might represent an important negative feedback control of CD40 functions.

The CD40/CD154 interaction is pivotal in the induction of both the humoral and cellular immune response and must therefore be strictly regulated. CD40 is a 50-kDa type I transmembrane protein that belongs to the tumor necrosis factor (TNF)[1]-receptor superfamily (for review see Refs. 1–3). It is expressed on the surface of antigen-presenting cells such as dendritic cells (4), B cells (5), and monocytes/macrophages (6), as well as in a broad spectrum of cell types, including endothelial cells (7), epithelial cells (8), and fibroblasts (9). The ligand for CD40 (CD154) is a 33-kDa type II transmembrane protein, a member of the TNF superfamily that is transiently expressed on the surface of activated T lymphocytes (10), predominantly of the $CD4^+$ subset. Basophils, mast cells (11), eosinophils (12), platelets (13), and natural killer cells (14) also express CD154. CD40 signaling triggered by CD154 is essential for B cell growth, differentiation, and immunoglobulin (Ig) class switching (15–17), as well as for antigen-presenting cell activation (18, 19), as it induces co-stimulatory molecule expression and cytokine synthesis (20, 21). It has been reported that upon ligation with CD40, CD154 is endocytosed and its mRNA expression is down-regulated (22). Although this mechanism is considered to be the major way the CD40/CD154 interaction is down-regulated, the production of a soluble form of CD40 (sCD40) could also be involved, because this molecule has been found in the supernatant of B lymphocytes co-cultured with activated T lymphocytes (22). Furthermore, we recently found evidence that sCD40 spontaneously produced by the lymphoblastoid JY cell line is functional, as it is able to bind membrane CD154 and to inhibit CD40/CD154-mediated Ig production by B cells *in vitro*,[2] demonstrating a potential antagonistic role for sCD40 in the immune response. However, little is known about the mechanism leading to sCD40 production.

Many cell-membrane proteins are subjected to limited proteolysis (called shedding) that gives rise to soluble forms consisting of the extracellular domain of the protein (for review see Refs. 23–26). The matrix metalloproteinase (MMP) family consists of structurally related enzymes that play an important role in tissue breakdown and remodeling during normal and pathological processes. Some MMPs have potential transmembrane protein cleavage activity, as demonstrated for matrylisin, which processes TNF-α (27) and Fas ligand *in vitro* (28). Among the proteases involved in membrane-anchored protein shedding, the metalloproteinases (MP) of the ADAM (for a disintegrin and MP) family are particularly important (for review see Refs. 29 and 30). Of the ADAM family members, the TNF-α converting enzyme (TACE) plays a critical role in the ectodomain shedding of pro-TNF-α (31, 32), TNF receptors I and II (33), L-selectin (34), CD30 (35), growth hormone receptor (36), macrophage colony-stimulating factor receptor (37), β-amyloid precursor protein (38), interleukin-6 receptor (24),

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‖ To whom correspondence should be addressed. Tel.: 33-5-57-57-14-71; Fax: 33-5-57-57-14-72; E-mail: julie.dechanet@umr5540.u-bordeaux2.fr.

[1] The abbreviations used are: TNF-α, tumor necrosis factor-α; sCD40, soluble CD40; mCD40, membrane-anchored CD40; MP, metalloproteinase; MMP, matrix metalloproteinase; ADAM, a disintegrin and metalloproteinase; TACE, tumor necrosis factor-α-converting enzyme (ADAM-17); FITC, fluorescein isothiocyanate; FCS, fetal calf serum; mAb, monoclonal antibody; TIMP, tissue inhibitor of metalloproteinase; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; MFI, mean fluorescence intensity.

[2] Contin, C., Pitard, V., Itai, T., Nagata, S., Moreau, J.-F., and Déchanet-Merville, J. (2003) *Immunology*, in press.

TNF-related activation-induced cytokine (39), fractalkine (40, 41), and the cellular prion protein (42).

The aim of the present study was to investigate the mechanism leading to sCD40 production by B lymphocytes. We provide evidence that the triggering of CD40 is sufficient to induce the rapid release of significant amounts of sCD40 and an associated decrease in the amount of membrane-anchored CD40 (mCD40) expression by either Epstein-Barr virus-transformed B cell lines or purified tonsil B cells. Finally, we demonstrated that the ADAM family member TACE cleaved a CD40-Fc chimeric molecule *in vitro* or mCD40 on the cell surface, leading to the production of its soluble counterpart. This processing might represent a way to both regulate the CD40 density at the cell membrane and generate a CD40 antagonist, ultimately achieving efficient negative feedback control of the CD40/CD154 interaction.

EXPERIMENTAL PROCEDURES

B Cell Isolation and Cell Lines

Total B cells were isolated from tonsils by T-cell rosetting as described previously (43). B cells were always >95% pure as assessed by flow cytometry with anti-CD19 fluorescein isothiocyanate (FITC) and anti-CD3 phycoerythrin (all from BD Biosciences, Marseille, France). The human B lymphoblastoid cell line JY and the Jurkat T cell line were maintained in RPMI 1640 medium (Invitrogen) supplemented with 8% fetal calf serum (FCS) (Amersham Biosciences) and 2 mM L-glutamine. HEK 293 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 8% FCS and 2 mM L-glutamine.

Antibodies and Reagents

The agonistic MAB89 anti-CD40 monoclonal antibody (mAb) (44) was a kind gift from Schering Plough Laboratory (Dardilly, France). Anti-CD40 3B2 and 10C6 mAbs, and anti-leukemia inhibitory factor 1F10 mAb (used as an isotype-matched irrelevant antibody) were produced in our laboratory. The non-blocking anti-CD40 FITC-conjugated mAb (clone EA-5) used to reveal mCD40 expression and the anti-TACE polyclonal antibody were both purchased from France Biochem (Meudon, France). The anti-actin polyclonal antibody was from Sigma.

Phorbol 12-myristate 13-acetate, brefeldin A, chloroquin, and monensin were from Sigma. The MP inhibitor GM6001 was purchased from France Biochem and the active recombinant tissue inhibitor of metalloproteinase-2 (TIMP-2) was a generous gift from Dr. A. Noël (Liège, Belgium). The inhibitor of furin convertase dec-RVKR-cmk was purchased from Alexis Biochemicals (Coger S.A, Paris, France) and the TACE peptide substrate (NH_2-LAQAVRSSSR-$CONH_2$) was produced by Cybergene (Evry, France).

Activation of B Cells with the Anti-CD40 mAb

Twenty four-well culture plates were coated with 10 μg/ml (unless otherwise stated) of anti-CD40 MAB89 or isotype-matched irrelevant control mAb overnight at room temperature in carbonate-bicarbonate buffer (0.2 M $NaHCO_3$, 0.8 M Na_2CO_3, pH 9.6). The wells were then washed once in phosphate-buffered saline (PBS) and then in RPMI 1640 without FCS. The JY cell line or tonsil B cells were washed in RPMI 1640 without FCS (to avoid inhibition of MP activity by the inhibitors present in the FCS), and plated (0.5×10^6 cells/well) in RPMI 1640 without FCS and supplemented with 2 mM L-glutamine, 5 IU/ml penicillin, and 5 μg/ml streptomycin (all from Invitrogen). The cells were then cultured for the indicated periods of time at 37 °C and 5% CO_2. Cell viability at the end of the culture period was always controlled by propidium iodide staining and flow cytometry analysis.

Enzyme-linked Immunoabsorbent Assay (ELISA) for the Detection of sCD40

Cell culture supernatants were spun for 10 min at 15,000 rpm to avoid contamination with the mCD40 that was associated with cell membrane fragments. The sCD40 level in samples was measured with an ELISA set up in our laboratory as follows. Maxisorp plates (Nunc, Merck, Strasbourg, France) were coated with the anti-CD40 MAB89 (1 μg/ml) in carbonate-bicarbonate buffer. The standards for the calibration curves were determined with recombinant sCD40 (consisting of the extracellular domain of CD40, amino acids 1–193 (22), produced in transfected COS cells and purified on a MAB89 affinity column). The binding of sCD40 was then revealed with the biotinylated 3B2 anti-CD40 mAb (0.5 μg/ml). Steptavidin peroxidase (Amersham, 1:1000) was used to develop the reaction with tetramethyl benzidine in phosphate-citrate buffer. Each sample was measured in duplicate and the mean concentration was calculated. The detection limit of the ELISA was around 3 pg/ml.

Flow Cytometry Analysis of mCD40 Expression and Total TACE Expression

Cells were harvested, washed in PBS, 0.1% bovine serum albumin, and stained for surface CD40 expression with a non-blocking FITC-conjugated anti-CD40 (clone EA-5) antibody able to recognize CD40 even in the presence of CD40-bound MAB89 (data not shown). The relative mean fluorescence intensity (relative MFI) was calculated with the following formula: (MFI (anti-CD40 FITC) − MFI (isotypic control Ab))/MFI (isotypic control Ab).

For the detection of total TACE expression, 10^6 tonsil B cells, JY, or HEK cells were fixed in 150 μl of fluorescence-activated cell sorter lysing solution (BD Biosciences), washed with PBS, and permeabilized with permeabilizing solution following the manufacturer's instructions (BD Biosciences). Cells were then stained for both membrane and intracytoplasmic TACE with an anti-TACE phycoerythrin-conjugated antibody (R&D systems, Abingdon, United Kingdom).

Cell-surface Protein Labeling Experiments

JY cells or tonsil B cells were washed twice in ice-cold PBS and subjected to cell-surface protein biotinylation with sulfosuccinimidobiotin (Pierce) according to the manufacturer's instructions. After extensive washes, the cells were resuspended in culture plates coated with MAB89 or control Ab. sCD40 was allowed to accumulate for 24 h in the culture supernatant that was then harvested and spun down for 10 min at 10,000 rpm. The cells were washed twice in ice-cold PBS and resuspended in lysate buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, supplemented with 1 mM phenylmethanesulfonyl fluoride and 2.5 μg/ml aprotinin (all from Sigma) for 30 min at 4 °C. Total cell lysate or culture supernatant were equally split for subsequent immunoprecipitation with Sepharose beads covalently coupled either with anti-CD40 10C6 mAb or control mAb. Immunoprecipitated materials were then run on a 10% SDS-polyacrylamide gel under reducing conditions, transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences), and the biotinylated proteins were revealed with streptavidin peroxidase (1:2000 final dilution). The positive bands were visualized using an ECL chemiluminescence kit (Amersham Biosciences).

Detection of TACE by Western Blot

Lysates were prepared from 10×10^6 cells (JY, tonsil B cells, or Jurkat cells) as described above. When mentioned, MP inhibitor GM6001 was added to the lysis buffer at 20 μM to enable the detection of either the pro- or the mature form of TACE, as previously described by Schlöndorff and colleagues (62). The insoluble material was then pelleted in a microcentrifuge at 15,000 rpm for 20 min at 4 °C, and the resulting supernatant was transferred to a fresh tube. Protein concentrations were determined using a bicinchoninic acid assay (Sigma). Normalized amounts of total protein (5 μg per lane) were loaded in SDS buffer under non-reducing conditions onto an 8% SDS-polyacrylamide gel, as required for TACE to be recognized by the antibody. Following electrophoresis, the proteins were electrotransferred onto a polyvinylidene difluoride membrane (Amersham Biosciences). The membrane was blocked in Tris-buffered saline with 0.1% Tween supplemented with 5% nonfat dried milk for 1 h at room temperature, then incubated with 5 μg/ml rabbit polyclonal anti-TACE or anti-actin antibody as indicated. After extensive washes in Tris-buffered saline with 0.1% Tween, the blots were probed with goat anti-rabbit (Zymed Laboratories Inc.) horseradish peroxidase-conjugated antibody, then washed to remove unbound material. The bands were visualized with an ECL chemiluminescence kit.

In Vitro CD40-Fc Cleavage Assay

The CD40-Fc chimeric protein (100 ng) comprising the extracellular domain of CD40, amino acids 1–193, fused to the Fc fragment of a human IgG1 (22), was incubated with 1 μg/ml recombinant TACE (R & D) with or without 100 μM GM6001 or TACE-specific substrate peptide for 5 h at 37 °C in 25 μl of total reaction mixture. The mixture was then fractionated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions. Recombinant sCD40 was also loaded as a control. After semi-dry transfer, the labeling was revealed with anti-CD40 H-10 (Santa Cruz Biotechnology, Santa Cruz, CA) antibody followed by sheep

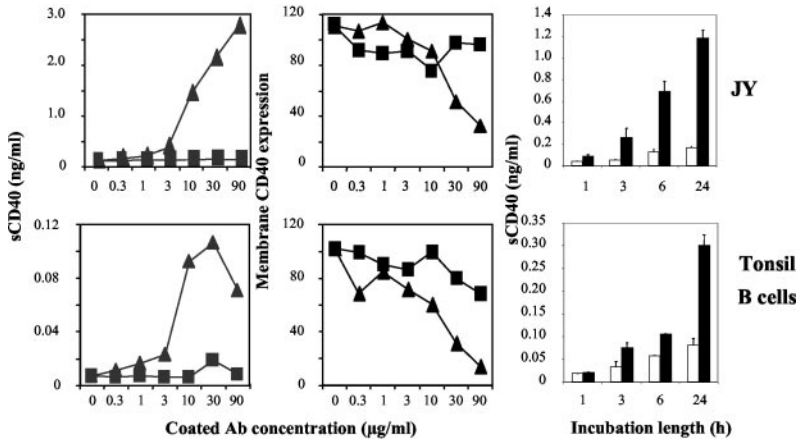

FIG. 1. **CD40 engagement induces sCD40 production and down-regulates surface CD40 expression.** 0.5×10^6 JY (*upper panels*) or 10^6 purified tonsil B cells (*lower panels*) were cultured in serum-free medium for 24 h in 24-well plates pre-coated with increasing concentrations from 0.3 to 90 μg/ml of anti-CD40 MAB89 (*triangles*) or an isotype-matched control antibody (*squares*). Supernatants were assessed by ELISA for the presence of sCD40 (*left panels*). mCD40 expression was revealed with a non-blocking FITC-conjugated anti-CD40 antibody EA5 (*middle panels*); the values in the *graphs* represent the relative mean of fluorescence intensity (MFI (EA5)-MFI (ctrl)/MFI (ctrl)). The kinetics of sCD40 production were assessed by ELISA of the supernatant of JY cells and tonsil B lymphocytes cultured in serum-free medium for 1, 3, 6, or 24 h in plates pre-coated with 10 μg/ml anti-CD40 (*filled bar*) or the isotype-matched control antibody (*white bar*). The *graphs* are all representative of at least three independent experiments.

anti-mouse horseradish peroxidase-conjugated antibody (Amersham Biosciences) and ECL.

Cell Transfection Experiments

Plasmid Constructs—The cDNA for CD40 has already been described elsewhere (22). The FLAG-tagged TACE and TACEΔM cDNA, respectively, encoding the full-length and a dominant-negative form of TACE lacking the metalloprotease catalytic domain, have also been previously described (45).

Transfection Experiments—Transfections were set up using the calcium-phosphate precipitation technique. Briefly, 4×10^5 HEK cells were seeded in plates with 6 flat-bottom wells, 24 h before transfection. Fresh medium was then added and the cells were incubated overnight at 37 °C with the transfection mixture: 0.5 or 0.25 μg of CD40 cDNA with or without 0.1–9 μg of TACE or TACEΔM cDNA in transfection medium (Hepes-buffered saline, pH 7.05, with 1 M $CaCl_2$). After 18 h of incubation, the medium was replaced with Dulbecco's modified Eagle's medium without FCS and sCD40 was allowed to accumulate in the culture medium for 48 h. The culture supernatant was then harvested and spun 15 min at 15,000 rpm.

RESULTS

CD40 Engagement on B Lymphocytes Induces sCD40 Release and Decreases the Amount of mCD40—The production of sCD40 was first reported in co-cultures of B lymphocytes with activated T cells (22). However, the molecular mechanism of this secretion was not investigated. To assess the possible role of CD40 engagement in sCD40 production by B cells, we activated Epstein-Barr virus-transformed B cells (JY cell line) or freshly isolated human tonsil B cells with an anti-CD40 mAb by culturing the cells in plastic plates that were coated with the mAb. After 24 h, the level of sCD40 in the supernatants was determined by a specific ELISA, and the expression of mCD40 was analyzed by flow cytometry.

As shown in the *left panel* of Fig. 1, the activation of JY cells and tonsil B cells by the anti-CD40 mAb (clone MAB89) induced sCD40 production in a dose-dependent manner, whereas an irrelevant isotype-matched mAb had no effect. As noted in previous studies (22, 46), we observed a minimal basal production of sCD40 (around 200 pg/ml) by non-activated JY cells, and much higher sCD40 levels secreted upon CD40 triggering by these cells than by tonsil B cells. In parallel, mCD40 staining was diminished on the surface of tonsil B cells and to a lesser extent on JY cells, when activated with coated anti-CD40 (*middle panels*). This was because of a decrease in the amount of mCD40 rather than to the masking of mCD40 detection by putative residual anti-CD40 mAb from the coating (MAB89), because the antibody used for cell labeling (clone EA5) recognized mCD40 even in the presence of MAB89 (data not shown). As shown in the *right panels* of Fig. 1, sCD40 production was induced within the first 3 h following activation with the coated anti-CD40 antibody in both B cell types. sCD40 release was associated with a prompt down-regulation of mCD40 staining (data not shown).

Soluble CD40 Is Generated through Proteolytic Cleavage of mCD40 by a Membrane-anchored MP—The mechanism of the production of the sCD40 form has not been known. A recent study suggested the CD40 mRNA might be alternatively spliced, leading to the expression of a truncated CD40 missing the intracellular and transmembrane domains (47). However, our results showing a rapid production of sCD40 upon activation and the concomitant disappearance of mCD40 pledged in favor of a direct shedding of CD40 from the cell surface. This latter process, unlike the *de novo* synthesis and secretion of a soluble truncated form of CD40, should not involve the intracellular transport of the soluble molecule and should thus be resistant to inhibitors of the Golgi and vesicular traffic. Accordingly, the addition for 6 h of brefeldin A, the weak amine chloroquin, or monensin to B lymphocytes activated by the anti-CD40 mAb did not affect sCD40 production (Fig. 2*A*). This result suggested that sCD40 production was the result of the direct shedding of CD40 from the cell surface. To formally demonstrate this hypothesis, JY cells were surface-labeled using sulfosuccinimidobiotin. As depicted in Fig. 2*B*, the engagement of cell-surface CD40 by MAB89 led to the specific recovery of a 28-kDa molecular species corresponding to the CD40 ectodomain, after the anti-CD40 was immunoprecipitated from the cell culture supernatant.

The proteolytic cleavage of numerous surface receptors is the result of the MP activity of the MMP or ADAMs family. We therefore next investigated the involvement of these enzymes in sCD40 regulation by testing the effect of two MP inhibitors in our culture model: GM6001, a broad spectrum synthetic inhibitor of MP, and recombinant TIMP-2, a natural inhibitor of MMP-2, which has also been shown to inhibit other MMPs, such as collagenase and gelatinase (48). As depicted in Fig. 3*A*, increasing doses of GM6001 inhibited the sCD40 production induced by CD40 ligation in both JY and tonsil B cells. Furthermore, the addition of GM6001 enabled the mCD40 expres-

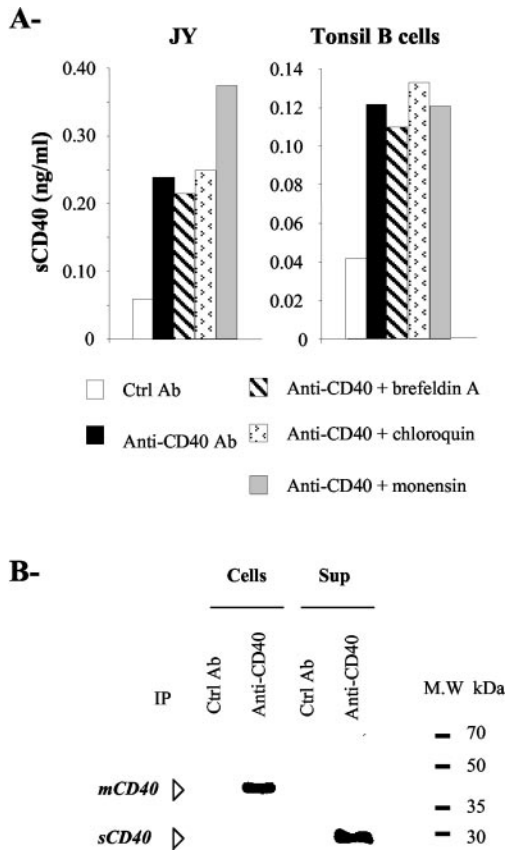

FIG. 2. **sCD40 is released by shedding.** *A*, JY cells or tonsil B cells were cultured for 6 h in 24-well plates pre-coated with anti-CD40 or isotypic control antibody, and in serum-free medium supplemented with 40 μM brefeldin A, 10 μM chloroquin, 10 μM monensin, or without supplement. sCD40 production was determined by ELISA. The values are the means of culture duplicates and are representative of three independent experiments. *B*, 2×10^6 biotinylated JY cells/ml were cultured for 24 h in serum-free medium in 6-well plates coated with anti-CD40 antibody. CD40 was immunoprecipitated from the cell lysates (*Cells*) or cell supernatants (*Sup*) with beads that were covalently coupled to anti-CD40 or an isotype-matched control antibody. Biotinylated immunoprecipitated materials were detected by Western blotting using streptavidin-conjugated horseradish peroxidase.

sion to return to basal levels in both cell types (Fig. 3*B*). The decrease in mCD40 expression after CD40 ligation and its inhibition by GM6001 was confirmed using cell-surface biotinylation experiments followed by a specific CD40 immunoprecipitation (Fig. 3*C*). In sharp contrast, TIMP-2 did not have any effect, either on sCD40 shedding or on mCD40 expression (Fig. 3, *A* and *B*), even at 200 μM, a concentration fully preventing the gelatinase activity of MMP-2 and MMP-9 in gelatin zymography assays (data not shown). Accordingly, stimulation of tonsil B or JY cells with anti-CD40 mAb coated on plastic never increased the levels of the MMP-2 or MMP-9 gelatinase activities found in culture supernatant, which is in contrast to their strong up-regulation by phorbol 12-myristate 13-acetate, which did not affect sCD40 production (data not shown). These results supported the idea that CD40 was cleaved proteolytically by an MP that was different from gelatinases and collagenases (data not shown).

Transmembrane MPs of both the membrane-type MMP (MT-MMP) and the ADAM family are converted to their active forms by an intracellular furin convertase of the paired basic amino acid-cleaving enzyme family (for review see Ref. 49). Peptidylchloromethyl ketones are competitive inhibitors of these convertases, and thus are specific inhibitors of transmembrane MPs. To determine whether CD40 shedding is mediated by one of these membrane-anchored MPs, we tested in a third step the capacity of dec-RVKR-cmk, one of the most effective inhibitors, to inhibit sCD40 release in our assay. When dec-RVKR-cmk was added to anti-CD40 activated B cells, sCD40 production was inhibited in a dose-dependent manner (Fig. 4), indicating that the shedding of CD40 from the B cell surface relies mainly on the enzymatic activity of a membrane-anchored MP.

TACE Is Able to Cleave Membrane-anchored CD40 and the Soluble CD40-Fc Chimeric Protein—One candidate putatively able to mediate CD40 shedding from the cell surface is TACE (ADAM-17), a transmembrane protease belonging to the ADAM family.

As a first step, we investigated whether TACE was expressed by B cells. Western blot (Fig. 5*A*) and flow cytometry (Fig. 5*B*) analyses revealed the constitutive expression of the 110-kDa pro-form of TACE by tonsil B cells and JY cells. In the Western blot experiment (Fig. 5*A*), the mature 80-kDa form of the MP was not detectable, probably because it was degraded during cell lysate preparation, as previously noted by Schlondörff and colleagues (62). However, the addition of GM6001 to cell lysates allowed the detection of the pro- (110 kDa) and mature (80 kDa) forms of TACE (Fig. 5*C*). Surprisingly, no modification in the expression of either form was seen during anti-CD40-induced sCD40 release in JY cells (Fig. 5*C*) and tonsil B cells (not shown) compared with the non-activated condition. Interestingly, pretreatment of JY cells with dec-RVKR-cmk hampered the expression of the mature form of the enzyme (80 kDa) and increased the expression of the pro-form (110 kDa) (Fig. 5*D*), confirming that this compound inhibits TACE processing and maturation, as previously described (37). Because dec-RVKR-cmk also abrogated sCD40 production (Fig. 4), this result suggests that TACE might be involved in the cleavage of mCD40.

To obtain further information about the putative implication of TACE in CD40 shedding, we next tested if CD40 could be cleaved by TACE in an *in vitro* setting. Recombinant TACE was incubated for 5 h at 37 °C with the CD40-Fc chimeric molecule, consisting of the Fc fragment of human IgG1 fused to the entire extracellular domain of CD40 (50). The mixtures were then fractionated by SDS-PAGE under reducing conditions and Western blotting was performed to detect sCD40. As depicted in Fig. 6, when CD40-Fc was incubated in the presence of recombinant TACE, a 28-kDa band appeared that corresponded to the size exhibited by recombinant sCD40. The addition of 100 μM GM6001 or 20 μM of a synthetic peptide cleaved by TACE (NH_2-LAQAVRSSSR-$CONH_2$) and used as a specific competitor completely inhibited CD40-Fc processing, confirming that TACE could process CD40.

We then tested the consequence of blocking the endogenous TACE activity on sCD40 release. To this end, we expressed in HEK 293 cells a dominant-negative form of TACE (TACEΔM), which lacks the catalytic MP domain (45), and was previously shown to prevent TACE-mediated TNF-α and TNFR I and II shedding (33, 45). HEK 293 cells, which constitutively expressed TACE as shown in Fig. 7*A*, also expressed the CD40-cleaving protease, because mCD40-transfected HEK cells spontaneously produced high levels of sCD40 (Fig. 7*B*). This production was abrogated in the presence of GM6001. HEK cells were then co-transfected with the cDNA coding for full-length mCD40 and with the cDNA coding for either TACE or TACEΔM. When mCD40 cDNA was co-transfected with increasing amounts of TACE cDNA, CD40 shedding was enhanced in a dose-dependent manner (Fig. 7*C*). Conversely, co-transfection with TACEΔM cDNA inhibited sCD40 release (Fig. 7*D*).

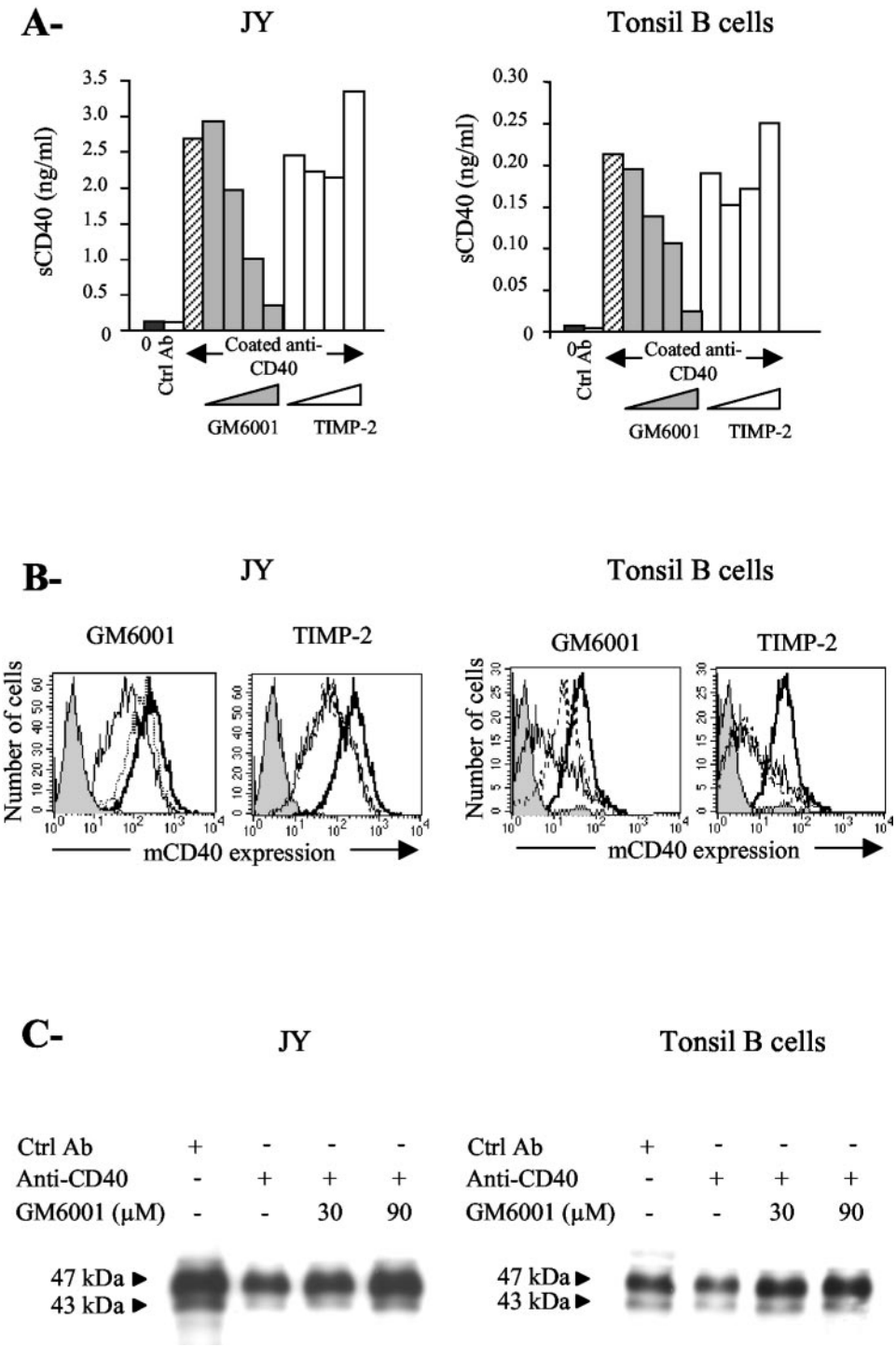

FIG. 3. **Inhibitors of MP can abrogate anti-CD40 engagement-mediated shedding.** *A*, JY cells (0.5×10^6/ml) or purified tonsil B cells (10^6/ml) were seeded in 24-well plates pre-coated with 10 μg/ml anti-CD40 or isotype-matched control antibody and were incubated for 24 h in serum-free medium in the presence of increasing concentrations of GM6001 (0.03, 0.3, 3, and 30 μM) or TIMP-2 (0.2, 2, 20, and 200 μM). sCD40 production in supernatant was then assessed by ELISA. The data are expressed as the mean of culture duplicates and are representative of at least three independent experiments. *B*, flow cytometry analysis of basal mCD40 expression (*thick line*), mCD40 expression in the presence of 10 μg/ml coated anti-CD40 with (*dotted line*) or without (*thin line*) 30 μM GM6001 or 200 μM TIMP-2. The *gray shaded* histogram represents labeling with the isotype-matched control mAb. *C*, membrane-biotinylated JY cells (0.5×10^6/ml) or tonsil B cells (10^6/ml) were incubated for 20 min at 37 °C with increasing amounts of GM6001 and stimulated for 6 h in 24-well culture plate with coated MAB89 or isotype-matched control mAb in the continuous presence of GM6001. Membrane CD40 was immunoprecipitated from cell lysates with 10C6 anti-CD40 mAb and biotinylated material was revealed with streptavidin-conjugated horseradish peroxidase (see "Experimental Procedures"). Immunoprecipitated mCD40 migrates as a doublet of 47 and 43 kDa as previously described by Braesch-Andersen *et al.* (73). The result shown is representative of three independent experiments.

DISCUSSION

The aim of the present study was to investigate the mechanism leading to sCD40 production. Although Van Kooten *et al.* (22) demonstrated that sCD40 is produced when B cells are co-cultured with activated T cells, little was known about the molecular factors inducing the production of this natural antagonist of the CD40/CD154 interaction. Here, we demonstrated that sCD40 production is induced after mCD40 ligation

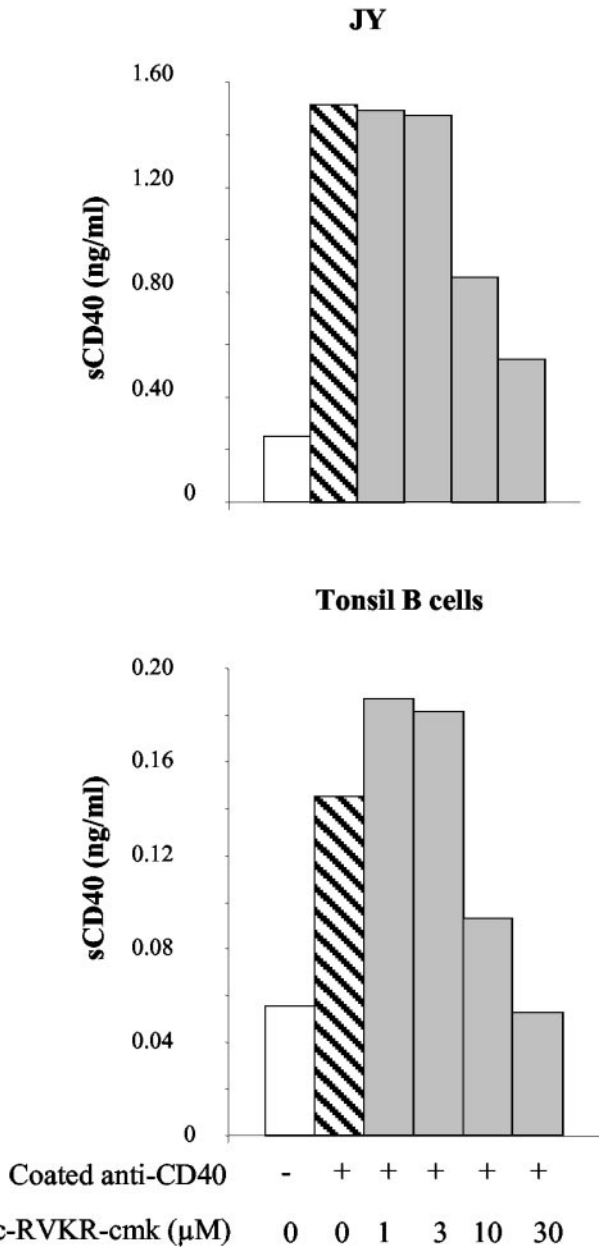

FIG. 4. **The CD40-cleaving protease is membrane-anchored.** JY cells (0.5 × 106) or purified tonsil B cells (10^6) were seeded in 24-well plates coated with 10 μg/ml anti-CD40 (*hatched bar*) or an isotype-matched control antibody (*open bar*) and were incubated for 24 h in serum-free medium in the presence of various concentrations of the furin convertase inhibitor dec-RVKR-cmk ranging from 1 to 30 μM (*gray bars*). The *graphs* show the means of duplicate cultures, and the data are representative of three independent experiments.

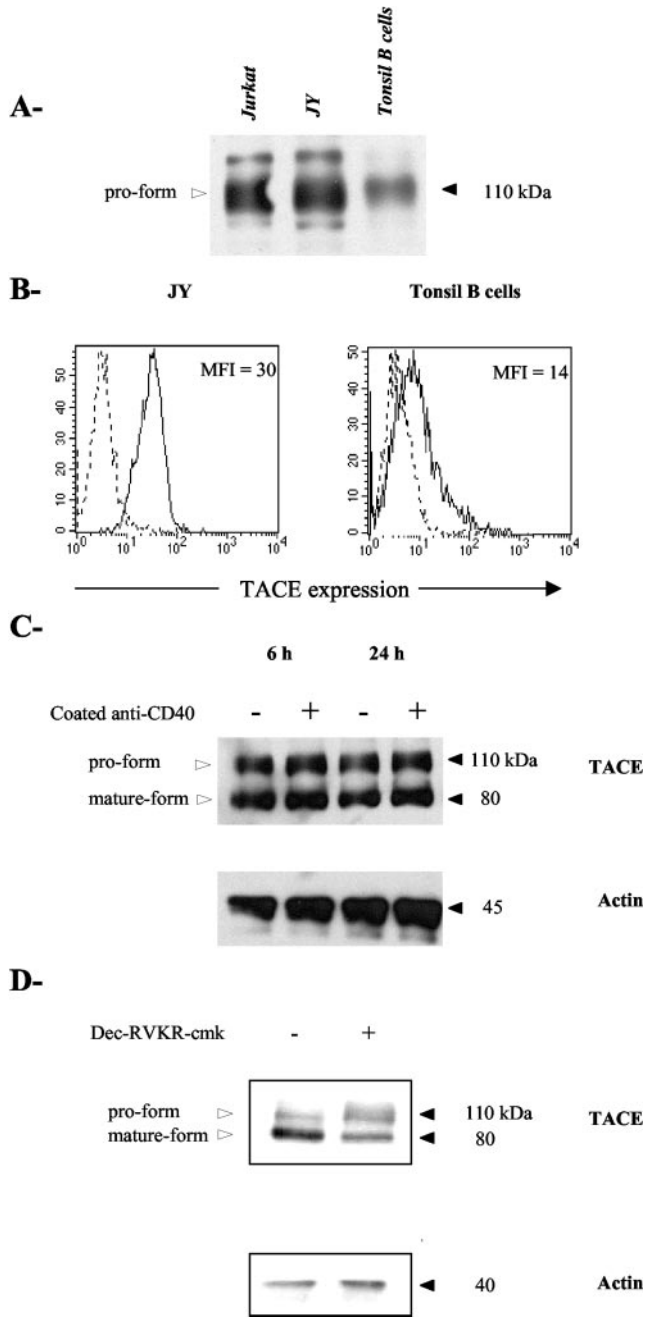

FIG. 5. **TACE expression and its regulation in JY and tonsil B cells.** *A*, TACE is expressed by JY and tonsil B cells. Cell lysates from Jurkat, JY, or tonsil B cells were tested for the presence of TACE by Western blotting as described under "Experimental Procedures." *B*, the total spontaneous TACE expression (*thin line*) was also tested for JY and tonsil B cells by flow cytometry (see "Experimental Procedures"). The *dotted line* represents the labeling with an isotype-matched control mAb. *C*, TACE expression is not modified during anti-CD40-induced sCD40 production. JY cells were cultured in serum-free medium in the presence of anti-CD40 or isotype-matched control antibody coated on plastic for the indicated periods of time. The cells were then harvested and lysed in the presence of GM6001 (20 μM) to allow the detection of the pro- (110 kDa) and the mature (80 kDa) form of TACE in Western blots as described under "Experimental Procedures." The blots were also probed with an anti-actin polyclonal antibody. *D*, inhibitors of furin convertase proteins hampered TACE processing in the JY cell line. JY cell cultures were incubated with or without 50 μM dec-RVKR-cmk for 24 h. The cells were harvested and lysed, and TACE or actin was detected by Western blotting as described under "Experimental Procedures."

and is associated with a diminution of mCD40 expression in both the Epstein-Barr virus-transformed JY cell line or purified tonsil B cells. The addition of inhibitors of intracellular and vesicular traffic did not alter sCD40 production, and cell-surface protein-labeling experiments confirmed that sCD40 produced in the culture supernatant of MAB89-activated JY cells resulted from cleavage of the mCD40 ectodomain. These results eliminated the possibility of the secretion of intracellular sCD40, generated, for instance, from the alternative splicing of CD40 mRNA. In contrast, in mice, alternative splicing of CD40 mRNA was described that produced a putative soluble form of CD40 (47). However, the same study also mentioned another probable mechanism of sCD40 production by cleavage of the mouse mCD40, generating a 27-kDa transmembrane fragment and a 18-kDa extracellular fragment. Our results provide evidence that CD40 cleavage occurs at the cell surface rather than in intracellular compartments. Both of these cleavage locations

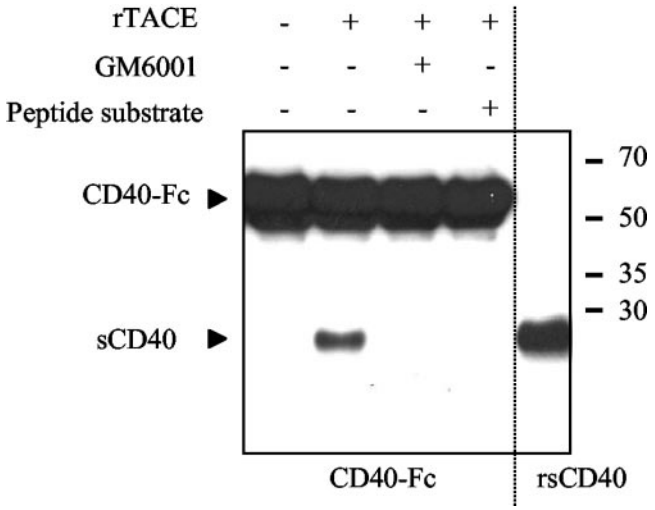

FIG. 6. **TACE can process the chimeric molecule CD40-Fc.** One hundred nanograms of CD40-Fc protein was incubated either alone or with 1 μg/ml recombinant TACE, and with 100 μM synthetic metalloprotease inhibitor GM6001 or 20 μM specific TACE peptide substrate, NH_2-LAQAVRSSSR-$CONH_2$, for 5 h at 37 °C in a 25-μl total reaction volume. Fifty nanograms of recombinant sCD40 was loaded in one lane as a control. Processed sCD40 and CD40-Fc were detected by Western blotting.

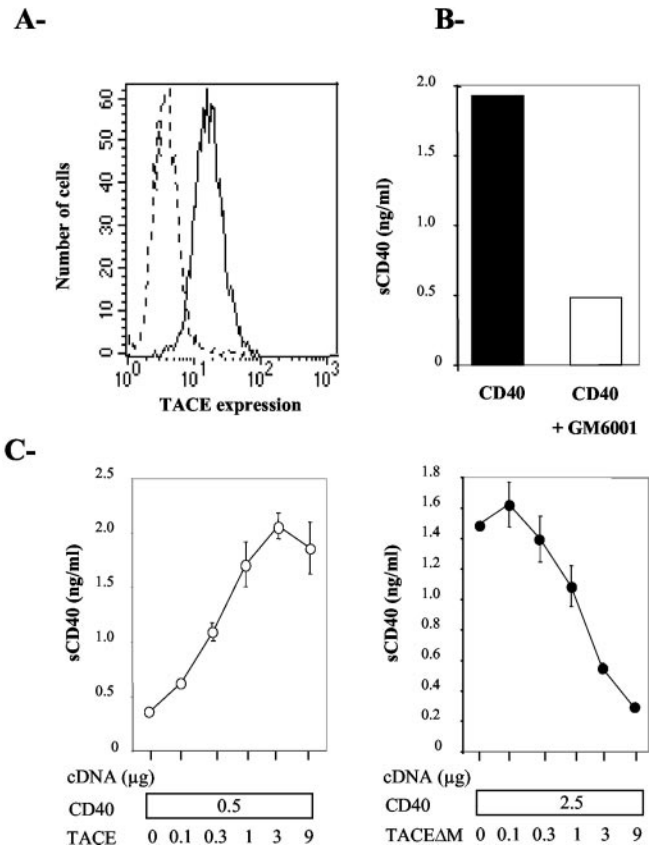

FIG. 7. **TACE is responsible for the shedding of membrane-anchored CD40.** *A*, TACE expression in HEK 293 cells. The spontaneous expression of TACE (intracytoplasmic and membrane) was determined by flow cytometry in HEK 293 cells (*thin line*). The *dotted line* represents labeling with the isotype-matched control. *B*, HEK cells express the CD40-cleaving protease endogenously. HEK cells transfected with 2.5 μg of plasmid encoding membrane-anchored CD40 were cultured for 48 h with or without 100 μM GM6001, and the amount of sCD40 in the supernatant was measured. *C*, the effect of co-transfecting TACE and TACEΔM on CD40 shedding. HEK cells were transfected with 0.5 (*C*) or 2.5 μg (*D*) of a plasmid encoding membrane-anchored CD40 with or without increasing amounts (0 to 9 μg) of plasmid encoding TACE (*C*) or TACEΔM (*D*). Cells were cultured in serum-free medium for 48 h, and sCD40 production was determined by ELISA of the culture supernatants. Data are expressed as the mean ± S.D. of three independent experiments.

have indeed been described for the shedding of transmembrane receptors. For instance, a close link was reported between the transport of TNF-α to the cell surface and its cleavage (33), whereas TGF-α is rapidly shed from the cell surface independent of vesicular traffic (51).

The process of shedding is important, as it up-regulates the production of soluble receptors that compete with the membrane receptor for ligand binding, and also reduces the amount of surface receptor, thus modulating the capacity of the cell to signal.

CD40 engagement has been reported to induce several extracellular matrix-degrading activities, such as MMP-1, MMP-2, MMP-9, and MMP-3 in the monocytic cell line THP-1, as well as peripheral blood monocytes and monocyte-derived macrophages (52, 53) and endothelial cells (54). However, the potential involvement of these MMPs in CD40 shedding is uncertain, insofar as TIMP-2, which inhibits MMP activity, had no inhibitory effect on CD40 shedding. In contrast, several lines of evidence have implicated TACE in CD40 shedding. First, it has been implicated in the shedding of other members of the TNF receptor family, namely CD30 (35) and TNF receptors I and II (33). Second, TACE-mediated TNF-α release is inhibited by broad spectrum metalloprotease inhibitors such as GM6001 (55, 56), but not by TIMP-2, as is sCD40 release in B cells. Third, we showed that TACE maturation is inactivated by dec-RVKR-cmk in B cells, thus confirming that its prodomain has to be removed by the action of a furin-type convertase in the late Golgi compartment to convert TACE to its active form (38, 57). The production of sCD40 by B cells is also inhibited by dec-RVKR-cmk. Although TACE expression has been well documented in monocytic lines, fibroblasts (31), and T lymphocyte lines (58), little was known about its expression in B lymphocytes. Here, we showed the constitutive expression of TACE in JY and human tonsil B cells equivalent to that exhibited by the T cell line Jurkat.

We then confirmed the involvement of TACE in CD40 shedding. First, *in vitro* cleavage assays of the chimeric protein CD40-Fc implicated a role for TACE in CD40 processing. Second, the overexpression of TACE in CD40-transfected HEK cells increased CD40 shedding, whereas co-expression of TACEΔM, a dominant-negative form of TACE, inhibited CD40 shedding in a dose-dependent manner. Similarly, the expression of TACEΔM in HEK cells transfected with TNF-α cDNA markedly inhibited TNF-α shedding (33, 45). However, the possible involvement of another protease in CD40 shedding cannot be excluded. Indeed, ADAM-10, which is closely related to TACE, has been described as processing TNF-α (59) and the β-amyloid precursor protein (60).

Flow cytometry and Western blot analyses did not show any increase in the total TACE expression in tonsil B cells and or JY cells, when incubated with anti-CD40 mAb coated on plastic. Similarly, neither the amount of TACE on the cell surface nor the extent of its processing activity are modified during the period of phorbol 12-myristate 13-acetate-induced shedding of TACE targets (31, 61). On the other hand, Schlöndorff and colleagues (62) did not show any alteration in the global membrane distribution of TACE during phorbol 12-myristate 13-acetate activation in COS-7 cells. It is possible that the engagement of mCD40 leads it to undergo a conformational change that allows its proteolytic cleavage by TACE, as this enzyme is more sensitive to conformation than to sequence. Another hypothesis is that CD40 engagement induced the relocation of CD40 into domains in which TACE usually resides, allowing

for the substrate to be cleaved. Indeed, the engagement of mCD40 induces its relocation into cholesterol-rich plasma membrane microdomains on the surface of B cells (63–66) and dendritic cells (67).

This better understanding of mCD40 regulation also offers a new perspective on emerging approaches to cancer therapies that involve activating anti-tumoral immunity. The use of an agonistic monoclonal antibody against CD40 or recombinant CD154 has emerged as one of the most effective ways to boost the immune response against infectious agents or to fight cancer (68–70). However, agonistic anti-CD40 antibody also induces immunosuppression in certain circumstances (71). Recently, Erickson *et al.* (72) showed in a murine model that heightened engagement of CD40 dramatically altered long-lived humoral immunity by inhibiting germinal center formation and the production of memory and long lived bone marrow plasma cells. Our results are consistent with this, and suggest that the constant activation of mCD40 by circulating antibody could induce abnormal CD40 shedding that could in turn down-regulate B cell activation. In conclusion, this study highlights a new mechanism for sCD40 production that promotes our understanding of the CD40-dependent regulation of B cell activation.

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