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Ceramide-Rich Membrane Rafts Mediate CD40 Clustering

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Many receptor systems use receptor clustering for transmembrane signaling. In this study, we show that acid sphingomyelinase (ASM) is essential for the clustering of CD40. Stimulation of lymphocytes via CD40 ligation results in ASM translocation from intracellular stores, most likely vesicles, into distinct membrane domains on the extracellular surface of the plasma membrane. Surface ASM initiates a release of extracellularly oriented ceramide, which in turn mediates CD40 clustering in sphingolipid-rich membrane domains. ASM, ceramide, and CD40 colocalize in the cap-like structure of stimulated cells. Deficiency of ASM, destruction of sphingolipid-rich rafts, or neutralization of surface ceramide prevents CD40 clustering and CD40-initiated cell signaling. These findings indicate that the ASM-mediated release of ceramide and/or metabolites of ceramide regulate clustering of CD40, which seems to be a prerequisite for cellular activation via CD40. The Journal of Immunology, 2002, 168: 298–307.
However, most, if not all, of these signaling events seem to depend on the clustering and recruitment of CD40 in sphingolipid-rich rafts, at least in dendritic cells (5) and B lymphocytes (6). Therefore, we aimed in the present study to define mechanisms mediating the aggregation of CD40 in rafts and the formation of a larger CD40 cluster. To this end, we investigated the role of the ASM and ceramide released in sphingolipid-rich membrane rafts for CD40 clustering.

Materials and Methods

Cells and stimulation

Human JY B lymphocytes, Niemann-Pick disease type A (NPDA) B lymphocytes, or Farber B lymphocytes were transfected with expression plasmids encoding ASM, vesicular stomatitis virus (VSV)-tagged ASM, Myc-tagged ASM, acid ceramidase (AC), or a control vector. NPDA cells were obtained from a patient with NPDA disease and Farber B cells were provided by Dr. K. Ferlinz (University of Bonn, Germany). To retransfect the cells, we subcloned cDNAs encoding unmodified ASM, VSV-tagged ASM, and AC into the pK vector. These subclones were designated pK-K-asm, pK-K-asm, and pK-K-asm, respectively (43, 44). The pH vector contains an additional gene that encodes a single-chain Ab fused with a Myc-tag to permit isolation of transfected cells by panning with the anti-Myc 9E10 Ab. In addition, the cDNA fragments encoding unmodified ASM and Myc-tagged ASM were subcloned into the pEF vector for expression as the Myc/EF fusion protein, respectively. Both pK and pEF vectors contain the elongation factor promoter to induce constitutive expression of ASM. Transient transfection of NPDA B lymphocytes was performed by using a BTX electroporator (GeneTec, San Diego, CA) that administered five 99-μs pulses at 550 V. Stably transfected cells were selected with 0.75 mg/ml G418, which was removed at least 24 h before any signaling studies were performed. Expression of ASM constructs was confirmed by flow cytometry and by measurements of ASM activity that indicated an expression level comparable to that in normal control JY B cells.

To disrupt sphingolipid- and cholesterol-rich rafts, we used 10 μM nystatin, 0.5 μg/ml filipin, or 1 mM of β-cyclodextrin. These drugs, which destroy sphingolipid-enriched rafts by disrupting cholesterol metabolism, were added 15 min before stimulation by anti-CD40. α-cyclodextrin (1 mM) was used as control. C20:4 arachidonic acid, sphingomyelin (SM), dihydro-C2-Ceramide, and dihydro-C24-Ceramide were dissolved in 0.1% octyl-glucopyranoside and used at 1 μM.

Fluorescence microscopy

For confocal and conventional fluorescence microscopy, lymphocytes were immobilized on glass coverslips coated with 1% (v/v) poly-l-lysine, washed, incubated with 100 ng/ml anti-CD40 5C3 Ab for 10 min at 4°C, washed again and stimulated for the indicated time with 1 μg/ml F(ab′)2 fragments of anti-mouse Ig (BD Pharmingen, San Diego, CA). Anti-ASM 9E10 Abs were obtained from Roche (Mannheim, Germany), the anti-ceramide 15B4 MAb was obtained from Alexis (Grunberg, Germany), choleratoxin and anti-choleratoxin Abs from Sigma-Aldrich (Deisenhofen, Germany), the anti-ASM antisera was kindly provided from Dr. K. Sandhoff (University of Bonn, Germany), washed three times with ASM lysis buffer, and lysed in 50 mM of Tris (pH 7.4), 1% Triton X-100, 1 mM of Na3 VO4, and 10 μg/ml soybean trypsin inhibitor, and 0.2% Triton X-100. They were then sonicated three times for 10 s each and the lysates were centrifuged for 2 min at 600 × g. ASM activity on the cell surface, whereas unstimulated cells displayed a homogenous distribution of the fluorescence throughout the membrane. In each experiment, the presence or absence of clustering in samples of 200 cells was scored by two independent observers. The results are given as the percentage of cells showing a cluster after the indicated time. All experiments were also performed without fixation in paraformaldehyde and revealed identical results.

Flow cytometry (FACS)

Cells in suspension were stained as described above using Cy3-labeled anti-ASM, Cy3-labeled anti-MyC-9E10 (1 μg/ml; Roche), Cy3-labeled anti-VSV (1 μg/ml; Roche) or Cy3-labeled anti-ceramide 15B4 (1/30 dilution), or isotype-matched irrelevant control mAbs and analyzed on a FACS Caliber (BD Biosciences, Franklin Lakes, NJ).

ASM activity

ASM activity was determined, as previously described (44, 45). In brief, cells were lysed in 50 mM of Tris (pH 7.4), 10 mM of bacitracin, 1 mM of dextrin, or α-cyclodextrin were added to the cells at concentrations of 100 ng/ml for 4 h at 4°C. The complexes were then incubated for 30 min at 37°C with [3H]serine (27 mCi/mM; Amersham). The lower phase was collected and radioactivity reflecting the degradation of [3H]ceramide was determined by liquid scintillation counting.

To analyze ASM activity on the cell surface, we stimulated 107 cells via CD40 ligation. We used a kit to biotinylate the cells according to the manufacturer’s instructions (Pierce, Rockford, IL). The cells were washed and lysed in 50 mM of Tris (pH 7.4), 1% Triton X-100, 1 mM of Na3VO4, and 10 μg/ml A/L. The complexes were then incubated for 30 min at 37°C with 50 mM of HEPES (pH 7.4), 2 mM of L-glutamine, 1 mM of sodium pyruvate, 100 mM of nonessential amino acids, 100 μM of penicillin, 100 μM of streptomycin, and 50 μM of 2-ME (Life Technologies, Karlsruhe, Germany). The mouse IgG1 anti-CD40 MAb (clone 5C3) was obtained from BD PharMingen (San Diego, CA). Anti-VSV PS54 and anti-MyC 9E10 Abs were obtained from Roche (Mannheim, Germany), the anti-ceramide 15B4 MAb was obtained from Alexis (Grunberg, Germany), choleratoxin and anti-choleratoxin Abs from Sigma-Aldrich (Deisenhofen, Germany), the anti-ASM antisera was kindly provided from Dr. K. Sandhoff (University of Bonn, Germany), washed three times with ASM lysis buffer, and lysed in 50 mM of sodium acetate (pH 5.0), 10 μM of Triton X-100, 1 mM of Na3 VO4, and 10 μg/ml A/L. The complexes were then incubated for 30 min at 37°C with [3H]serine (5.0 μCi/sample, 54.5 Ci/mM; NEN, Boston, MA) in 250 mM of sodium acetate (pH 5.0), 1.3 mM of EDTA, and 0.05% Nonidet P-40. Samples were extracted with a mixture of CHCl3, CH2OH (dilution, 2/1), and H2O. The upper phase was collected and radioactivity reflecting the degradation of [3H]ASM was determined by liquid scintillation counting.

To analyze ASM activity on the cell surface, we stimulated 107 cells via CD40 ligation. We used a kit to biotinylate the cells according to the manufacturer’s instructions (Pierce, Rockford, IL). The cells were washed and lysed in 50 mM of Tris (pH 7.4), 1% Triton X-100, 1 mM of Na3VO4, and 10 μg/ml A/L. The lysates were subjected to precipitation with streptavidin-coupled agarsose and the streptavidin precipitates were then analyzed for ASM activity, as described above.

Ceramide release

Cells were labeled for 48 h with 2 μCi/ml [3H]serine (27 mCi/mM; NEN). Cells were then stimulated via CD40 ligation and ceramide was extracted in a solution of CHCl3, CH2OH, H2O, and piridine (dilution, 60/40/160/6). The lower phase was collected and the ceramides contained therein were degraded with methanolic NaOH at 37°C for 2 h. Samples were re-extracted in a solution of CHCl3, CH2OH, and 1 M of HCl (dilution, 100/100/1), dried, and resuspended in a solution of CHCl3 and CH2OH (dilution, 95/5). Lipids were separated on silica G60 thin-layer chromatography plates (Merck, Whitehouse Station, NJ) by using a solution of CHCl3, CH2OH, and CH2OH (dilution, 60/60/60). Lipids were then dried and the substances that migrated to the same position as the radioactive [3H]ceramide standard was identified as ceramide. Results obtained with [3H]serine labeling were confirmed with the 1,2-diacylglycerol kinase method (not shown).

IL-12 assays

JY, ASM, or control-transfected NPDA cells were cultured in RPMI 1640 supplemented with 1% FCS and stimulated via CD40 using 100 ng/ml plate-bound anti-CD40 for the indicated time. Nystatin, filipin, β-cyclodextrin, or α-cyclodextrin were added to JY cells 30 min before stimulation via CD40. Supernatants were collected and the release of IL-12 was determined by ELISA exactly following the instruction of the vendor (R&D Systems, Minneapolis, MN).
JNK activity

JY cells and ASM-deficient or reconstituted NPDA lymphocytes were stimulated for 20 min, lysed in 25 mM of HEPES (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 125 mM of NaCl, 10 mM each of NaF, Na3VO4, and sodium pyrophosphate and 10 μg/ml A/L. JNK was immunoprecipitated from the postcentrifugation lysates for 4 h at 4°C using polyclonal rabbit anti-human JNK antiserum (Santa Cruz Biotechnology). Immunocomplexes were immobilized on protein A/G (Santa Cruz Biotechnology), incubated for an additional 60 min at 4°C, washed twice in lysis buffer, twice in 132 mM of NaCl, 20 mM of HEPES, 5 mM of KCl, 1 mM of CaCl2, 0.7 mM of MgCl2, 0.8 mM of MgSO4, 1% Nonidet P-40, 2 mM of Na3VO4, once in 100 mM of Tris (pH 7.5), 0.5 M of LiCl, and, finally, twice in kinase buffer consisting of 12.5 mM of MOPS (pH 7.5), 12.5 mM of β-glycerophosphate; 0.5 mM of EGTA, 7.5 mM of MgCl2, 0.5 mM of NaF, 0.5 mM of Na3VO4. Samples were finally resuspended in kinase buffer supplemented with 10 μCi/sample [γ-32P]ATP (6000 Ci/mmol; NEN; DuPont Pharmaceuticals, Wilmington, DE), 10 μM of ATP, and 1 μg/ml GST-c-Jun (aa 1–79), incubated at 30°C for 15 min and the kinase reaction was stopped by addition of 5 μl of boiling 5× reducing SDS sample buffer. Samples were separated by 10% SDS-PAGE and analyzed by autoradiography. The substrate GST-c-Jun was expressed in DH5α-bacteria and purified via glutathione agarose.

FIGURE 1. CD40 clusters upon stimulation. Stimulation of JY B lymphocytes via CD40 ligation resulted in rapid CD40 clustering, as indicated by confocal fluorescence microscopy. Shown is a typical time course (A) and a quantitative analysis (B) of data from five experiments, which each included analysis of 200 cells/time point. Given is the percentage of cells with a membrane cluster at a given time. The specificity of CD40 clustering was evidenced by the absence of clustering of other receptors including the BCR, CD95, L-selectin, or β2 integrin (C). Fluorescence microscopy of cells that had been stimulated via CD40 and stained with Cy3-labeled anti-CD95, anti-L-selectin, or anti-β2 integrin demonstrate the specificity of CD40 clustering (D). E, Confocal microscopy studies reveal a colocalization of clustered CD40 with Cy5-choleratoxin (cholera) indicative for clustering of CD40 in sphingolipid-rich rafts.
Results

ASM mediates CD40 clustering

To determine whether stimulation via CD40 ligation results in clustering of the receptor, we stimulated JY B cells by using cross-linked anti-CD40 Abs. Small clusters of CD40 formed on the cell surface within a few seconds (Fig. 1, A and B). Within minutes of stimulation, these clusters enlarged and formed cap-like structures. The specificity of this clustering process is evident, because stimulation via CD40 ligation induced exclusive clustering of the cognate receptor (Fig. 1, C and D), but not of other receptors, including CD95, BCR, L-selectin, or β2 integrin. Stimulation with a very high concentration of anti-CD40 (2 and 20 μg/ml secondary F(ab')2) resulted in the formation of cluster-like structures on almost all cells (92 ± 6%). Clustering of CD40 occurred in cholera toxin-positive rafts (Fig. 1E). Cholera toxin binds to GM1 gangliosides highly enriched in sphingolipid-rich rafts suggesting that CD40 clusters in those rafts.

To assess whether membrane changes affect receptor clustering, we investigated the role of ASM in CD40 clustering. To this end, we stimulated JY B cells via CD40 ligation and determined the activity of ASM and the release of ceramide. ASM activity rapidly increased ~2-fold upon CD40 ligation (Fig. 2A) correlating with a release of ceramide (Fig. 2B).

To investigate the function of ASM in CD40 clustering, we used human NPDA B lymphocytes, which are genetically deficient of ASM. The cells were either transfected with the ASM expression vector pJK-asm (NPDA/ASM+/+) or with the control vector pJK...
(NPDA/ASM\(^{-/-}\)). ASM deficiency prevented CD40 clustering after stimulation via CD40 ligation; however, CD40 clustering was restored upon transfection of the ASM expression vector (Fig. 2C). ASM deficiency did not result in a general defect of receptor clustering, because clustering of L-selectin, which does not activate ASM (46), occurred in the NPDA/ASM\(^{-/-}\) cells and the NPDA/ASM\(^{+/+}\) cells (Fig. 2C).

To further investigate the role of ceramide in receptor clustering, we tested CD40 clustering in AC-deficient B cells. AC metabolizes ceramide and thus may function as a negative regulator of ceramide activity. AC-deficient cells showed a marked increase in CD40 clustering (Fig. 2D); the level of clustering in AC-deficient cells transfected with the AC expression vector was similar to that seen in control B cells. These results indicate a role for ceramide in CD40 clustering, however, they do not exclude that some ceramide metabolites are also involved in CD40 clustering.

**CD40 stimulation induces translocation of ASM onto the extracellular leaflet of the cell membrane**

To gain insight into the mechanisms of ASM-mediated CD40 clustering, we investigated the subcellular distribution of ASM and whether ASM colocalizes with clustered CD40. FACS studies demonstrated a translocation of ASM from an intracellular location in unstimulated cells onto the cell surface after CD40-mediated stimulation (Fig. 3A). Permeabilization of the cells with 0.1%

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**FIGURE 3.** ASM translocates onto the cell membrane and colocalizes with clustered CD40. Stimulation via CD40 ligation resulted in translocation of the ASM onto the extracellular leaflet of the cell membrane (A), as determined by FACS of permeabilized or intact JY B lymphocytes. Unstimulated cells showed almost no surface ASM. FACS analyses of intact JY cells that had been transfected with pJK, pJK-asm, pJK-myc-asm, or pJK-ves-asm showed a marked increase of the Cy3 signal upon stimulation via CD40 ligation (B) upon staining with Cy3 anti-ASM, Cy3 anti-Myc 9E10, or Cy3 anti-VSV P5D4, observations that indicated translocation of ASM onto the cell surface upon stimulation of CD40 and confirmed the specificity of ASM detection. Immunoprecipitation of biotinylated surface proteins from stimulated or unstimulated JY B cells confirmed the translocation of functional ASM onto the cell surface (C). Confocal microscopy of JY B cells revealed clustering of CD40 (D, left), a translocation of the ASM onto the surface of the cell membrane (D, middle) and colocalization of ASM with clustered CD40 upon stimulation (D, right). Intact cells were stained with Cy3-coupled anti-CD40 5C3 Abs (left column) and Cy5-labeled anti-ASM Abs (middle column). ASM was not detectable on the surface of unstimulated cells. On the right, the two pictures were superimposed. The fluorescence signals between the fluorochrome detection channels did not overlap. Irrelevant Abs did not cross-react with the cells. Shown is a representative result of five similar independent experiments that each included analysis of 100 cells.
Triton X-100 reveals that 10–15% of the cellular ASM translocates to the cell surface upon stimulation. The specificity of our detection of ASM was demonstrated by transfection of Myc-tagged ASM and staining with Cy3-labeled anti-Myc 9E10 Ab and by transfection with VSV-tagged ASM and staining with anti-VSV P5D4 Ab (Fig. 3B). To further confirm the translocation of functional ASM, we biotinylated surface proteins and measured ASM activity in streptavidin-selected immunoprecipitates. These studies revealed high ASM activity on the surface of stimulated cells but not of unstimulated cells (Fig. 3C).

Confocal microscopy revealed that surface ASM and CD40 colocalized within the cluster region, whereas unstimulated cells displayed a homogenous distribution of CD40 and lacked surface ASM (Fig. 3D).

Together, these results indicate that activated CD40 clusters and colocalizes with ASM, which translocates onto the extracellular surface of the plasma membrane upon stimulation.

**Surface ceramide triggers clustering of activated CD40**

On the basis of our results, we hypothesized that ASM generates extracellularly oriented ceramide upon stimulation of CD40. To test this hypothesis, we stained JY B lymphocytes before or after stimulation of CD40 with Cy3-labeled anti-ceramide 15B4 Ab. FACS showed that a substantial amount of the anti-ceramide Ab bound to the stimulated cells but not to unstimulated cells (Fig. 4A). CD40 and ceramide colocalized in the cluster-like structure upon stimulation of the cells via CD40 (Fig. 4B). Unstimulated cells almost completely lacked surface ceramide. The importance of extracellularly oriented ceramide for CD40 clustering is indicated by the abrogation of CD40 clustering upon neutralization of surface ceramide by the anti-ceramide 15B4 mAb (Fig. 4C) or the destruction of sphingolipid-rich rafts by β-cyclodextrin, nystatin, or filipin (Fig. 4D). The inactive stereoisomer α-cyclodextrin did not affect CD40 clustering. Binding of the Ab to ceramide very

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**FIGURE 4.** Surface ceramide mediates CD40 clustering. A, Activation of JY B cells via CD40 ligation resulted in a release of extracellularly oriented ceramide, as evidenced by FACS analysis of cells stained with anti-ceramide 15B4 before or after stimulation. B, Surface ceramide colocalized with clustered CD40 upon stimulation as evidenced by confocal microscopy studies. Shown is a representative result from three independent experiments with the analysis of 100 cells each. C, Neutralization of extracellularly oriented ceramide on the cell surface by incubation of the cells with 200 ng/ml anti-ceramide 15B4 prevents clustering of CD40 determined by fluorescence microscopy. D, Destruction of rafts using β-cyclodextrin, nystatin, or filipin prevents CD40 clustering upon stimulation. The inactive stereoisomer α-cyclodextrin did not alter CD40 clustering. C and D, The mean ± SD of three independent experiments with analysis of 200 cells each.
likely alters the biophysical properties, e.g. the mobility, of the lipid and/or the ability of ceramide to interact with other lipids or proteins and, thus, the Ab is able to prevent the formation of receptor clusters.

Next, we investigated whether the generation of surface ceramide is sufficient to initiate CD40 clustering. When cells were treated with 100 ng/ml of anti-CD40 5C3 Ab that was not cross-linked, CD40 clustering did not occur (Fig. 5). Combinations of noncross-linked 5C3 Ab and pure ASM or the Ab and natural C\textsubscript{16}-ceramide were sufficient to initiate CD40 clustering in NPDA/ASM\textsuperscript{+} cells and to restore clustering in NPDA/ASM\textsuperscript{−} cells (Fig. 5). Cross-linking of 100 ng/ml anti-CD40 5C3 Ab by a secondary F(ab')\textsubscript{2} (1 µg/ml) also initiated CD40 clustering in ASM-expressing cells, but not in ASM-deficient B lymphocytes (Fig. 5). The addition of the 5C3 Ab, pure ASM, or C\textsubscript{16}-ceramide alone did not induce CD40 clustering. Likewise, CD40 clustering was not induced by treatment of the cells with 5C3 anti-CD40 Abs and any of the following agents: arachidonic acid (C\textsubscript{20:4}), SM, dihydro-C\textsubscript{16}-ceramide, or dihydro-C\textsubscript{16}-ceramide. A very high dose of anti-CD40 Abs (2 µg/ml + 20 µg/ml secondary F(ab')\textsubscript{2}) restored clustering of CD40 (Fig. 5) in ASM-deficient cells, which is very likely due to the forced formation of CD40 cluster by Ab aggregates.

These results support the notion that ceramide is necessary and sufficient for the clustering of activated CD40.

**ASM-mediated CD40 clustering is required for CD40 signaling**

We tested whether the activation of signaling events depends on clustering of CD40. Genetic deficiency of ASM, treatment with anti-ceramide Abs or destruction of rafts prevented CD40-mediated release of IL-12 (Fig. 6, A and B). These findings indicate that CD40 clustering in ceramide-rich rafts is important for CD40-mediated cellular signaling. The failure of ASM-deficient cells to release IL-12 was dose-dependent and high doses of plate-bound anti-CD40 Abs restored, at least partially, IL-12 release (Fig. 6C). Treatment of the cells with β-cyclodextrin, nystatin, or filipin did not affect surface translocation of the ASM (Fig. 6D) or activation (not shown) by CD40 indicating that signals occurring before CD40 clustering are not altered by the drugs.

To further indicate the significance of ASM-mediated clustering of CD40, we determined JNK activity in ASM-deficient or constituted NPDA cells or in cells treated with β-cyclodextrin or the inactive isomer α-cyclodextrin. ASM-deficient cells failed to activate JNK upon stimulation via CD40, while retransfecation of ASM restored JNK activation (Fig. 6E). Likewise, treatment of the cells with β-cyclodextrin abrogated CD40-mediated JNK activation, whereas α-cyclodextrin was without effect.

**Discussion**

Our data suggest a novel function of ceramide and the ASM, i.e., the mediation of CD40 clustering, which seems to be a critical event in transmembrane signaling through this receptor. Because we have previously demonstrated a similar function of ceramide and the ASM for CD95 (9, 10) and preliminary data also extend that function of ceramide and the ASM to CD48 and CD28 signaling (E. Gulbins, unpublished results) as well as to infection of some pathogenic bacteria into mammalian cells (9, 44, 47), the observed function of the ASM might be operative for many receptor systems.

Our data suggest the following model of ASM-mediated clustering of CD40: primary stimulation via CD40, which seems to be insufficient to trigger complete cellular activation, induces an activation and membrane translocation of the ASM from an intracellular compartment, most likely vesicles, to the cell membrane. Fusion of ASM-containing vesicles with the cell membrane results in exposure of the ASM to the extracellular surface. ASM causes a localized release of extracellularly oriented ceramide and this molecule may function in several ways in the clustering process. First, ceramide has been shown to self-aggregate (48–50). A self-aggregation of ceramide in pre-existing sphingolipid-rich rafts may generate very small primary signaling microdomains. Second, because ceramide-rich microdomains spontaneously fuse (51), the generation of ceramide in those sphingolipid-rich rafts may trigger the fusion of those very small entities into larger membrane domains. The fusion of very small microdomains triggers the clustering of CD40 molecules already present in those rafts. Previous studies on dendritic cells and B lymphocytes (5, 6), as well as preliminary data from our lab, indicate a substantial amount of CD40 in Triton X-100 insoluble membrane fractions already present before receptor stimulation suggesting that ceramide-mediated fusion of small rafts to larger platforms constitutes a major mechanism to cluster CD40. In addition, ceramide may also gather CD40 molecules into membrane rafts, prevent diffusion of CD40 molecules from rafts, or function in both ways. The findings from our studies on AC-deficient Farber lymphocytes as well as those from studies that included anti-ceramide 15B4 Ab binding strongly support a crucial role of ceramide in CD40 clustering. However, we cannot exclude the possibility that some metabolite(s) of ceramide, e.g., glycosylated ceramides, are involved in clustering, because some ceramide metabolites may also accumulate in Farber lymphocytes upon stimulation and may be detected by the anti-ceramide Abs. The FACS analysis and fluorescence microscopy examinations presented in this study revealed that the anti-ceramide Ab 15B4 did not bind to unstimulated cells, whereas binding occurred rapidly after stimulation of CD40. In addition, ASM-deficient cells failed to bind the Ab even after stimulation.

**FIGURE 5.** Ceramide is required and sufficient to induce clustering of CD40. Low-dose anti-CD40 5C3 Ab (100 ng/ml) that was not cross-linked did not induce CD40 clustering in cells expressing or lacking the ASM. Simultaneous addition of pure ASM or C\textsubscript{16}-ceramide and anti-CD40 5C3 Ab was sufficient to restore CD40 clustering even in cells lacking ASM. F(ab')\textsubscript{2}-mediated cross-linking of an anti-CD40 5C3 Ab induced clustering in ASM-expressing cells and served as positive control. Application of a very high dose of cross-linked CD40 (2 µg/ml anti-CD40 + 20 µg/ml secondary F(ab')\textsubscript{2}) resulted in CD40 clustering even in ASM-deficient cells. None of the indicated controls induced clustering. Clustering was determined by fluorescence microscopy (n = 5, mean ± SD; analysis of 200 cells each).
FIGURE 6. CD40 clustering is required for CD40 signaling. Genetic deficiency of ASM, anti-ceramide 15B4 Ab-mediated neutralization of surface ceramide (A), or destruction of rafts by β-cyclodextrin, nystatin, or filipin (B) prevented release of IL-12 upon 24 h stimulation with plate-bound CD40. The inactive stereoisomer α-cyclodextrin had no effect. None of the reagents reduced viability of the cells and all samples showed ~2–3% dead cells after the 24 h incubation. To assess the effects of β-cyclodextrin, nystatin, filipin, or α-cyclodextrin, the fetal bovine concentration was reduced to 2.5%. All reagents were added 30 min before initiation of CD40 stimulation and re-added after a 12-h incubation period. Given is the mean ± SD of three independent experiments. C, High doses of plate immobilized anti-CD40 (10 μg/ml) restore IL-12 release from ASM-deficient cells, while lower doses require the ASM for efficient IL-12 release. ASM-deficient or reconstituted NPDA were incubated for 24 h with the indicated concentration of plate-bound CD40. IL-12 release was determined by ELISA. Given is the mean ± SD of three independent experiments. High concentrations of plate-bound anti-CD40 very likely induce an artificial CD40 clustering abrogating the requirement of ASM for cell endogenous clustering. D, FACS analysis shows that treatment of JY cells with β-cyclodextrin, nystatin, or filipin does not alter the translocation of the ASM onto the cell surface upon stimulation via CD40, while lower concentrations of anti-CD40 require ASM expression for clustering and signaling. Shown is a representative analysis for three experiments. E, Deficiency of the ASM or destruction of rafts using β-cyclodextrin abrogates JNK activation upon CD40 triggering. Retransfection of the ASM restores JNK stimulation, the inactive isomer α-cyclodextrin was without effect on JNK stimulation. Nonspecific (n.s.) immunoprecipitates were performed with an irrelevant rabbit Ig and were negative. An aliquot of all immunoprecipitates was blotted with anti-JNK to confirm similar loading in all lanes. Shown is a representative blot from three independent kinase assays.
with anti-CD40 Ab. This result excludes the possibility of a substantive reaction with cholesterol, SM, or phospholipids. The observation that loading intact cells with C16-ceramide resulted in substantial Ab binding to the cell surface provides additional evidence of a specific detection of ceramide. Also, the anti-ceramide Ab detected a single band on high performance thin layer chromatography plates containing lipid extracts from CD40-stimulated cells and this band migrated to the same position as C16-ceramide. The anti-ceramide Ab did not detect dihydro-C16-ceramide or SM (E. Gubins, unpublished data).

In the present study, we used several approaches to determine ASM translocation onto the cell surface: the specificity of these findings was confirmed by Ab staining of cells transfected with Mmc- or VSV-tagged ASM constructs. In addition, the anti-ASM Ab failed to bind to B lymphocytes derived from ASM knockout mice, whereas B lymphocytes from normal control mice showed a rapid surface translocation of ASM upon CD40 ligation (E. Gubins, unpublished observations).

Clustering of CD40 in rafts may serve many functions. First, multimerization of CD40 may permit the translocation of molecules associated with CD40, e.g., JAK3 (34), even if only a few dispersed molecules are primarily active. Second, translocation of CD40 may bring the receptor into close contact with signaling molecules present in rafts and may permit activation of these signaling molecules by CD40. Third, inhibitory signaling molecules, such as the tyrosine phosphatase CD45 (4, 22), might be excluded from rafts, a situation that may further enhance signaling via CD40. Fourth, immobilization of CD40 in a defined membrane domain may stabilize the interaction of CD40 with its ligand. Lateral diffusion of raft-trapped CD40 might be greatly reduced; thus, the presence of CD40 in rafts may prolong binding to its ligand.

In conclusion, CD40 ligation induces activation and surface translocation of ASM, which colocalizes with sphingolipid-rich rafts. The activity of ASM causes the localized release of extracellularly oriented ceramide in sphingolipid-rich rafts, which in turn mediates clustering of CD40. Finally, CD40 clustering in ceramide-enriched rafts is required for the initiation of signaling within cells.

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References


