Receptor clustering is a key event in the initiation of signaling by many types of receptor molecules. Here, we provide evidence for the novel concept that clustering of a ligand is a prerequisite for clustering of the cognate receptor. We show that clustering of the CD40 receptor depends on reciprocal clustering of the CD40 ligand (gp39, CD154). Clustering of the CD40 ligand is mediated by an association of the ligand with p53, a translocation of acid sphingomyelinase (ASM) to the cell membrane, an activation of the ASM, and a formation of ceramide. Ceramide appears to modify preexisting sphingolipid-rich membrane microdomains to fuse and form ceramide-enriched signaling platforms that serve to cluster CD40 ligand. Genetic deficiency of p53 or ASM or disruption of ceramide-enriched membrane domains prevents clustering of CD40 ligand. The functional significance of CD40 ligand clustering is indicated by the finding that clustering of CD40 on B lymphocytes upon co-incubation with CD40 ligand-expressing T cells depends on clustering of the CD40 ligand and is abrogated by inhibition of CD40 ligand clustering.

Receptor clustering or aggregation is a central event in the signaling of many types of receptor molecules and is initiated by the interaction of a ligand with its cognate receptor. If the ligand is membrane-bound, the contact site between the ligand and the receptor resembles some features of a neurological synapse and, thus, has been also named immune synapse (1).

Many receptors appear to aggregate in sphingolipid-rich membrane rafts, which contain a high concentration of sphingolipids and cholesterol (2–4). The biophysical properties of these membrane domains cause them to separate from the phospholipids in the cell membrane and to resist breakdown by some detergents (2–4). Therefore, these small rafts were also named detergent-insensitive, glycosphingolipid-rich domains. Most studies on receptor clustering focused on the role of intracellular signaling molecules (for recent reviews, see Refs. 5 and 6); however, we have recently suggested that membrane changes also contribute to receptor clustering (7–10). These studies revealed that stimulation of CD95 or CD40 triggers the fusion of acid sphingomyelinase (ASM)-containing, intracellular vesicles with the cell membrane (7, 9, 10). This fusion results in the exposure of the enzyme to the extracellular leaflet of the cell membrane. ASM activity results in the formation of extracellularly oriented ceramide from sphingomyelin (7, 9, 10). Surface ceramide seems to reorganize small lipid rafts into larger platforms, which serve to trap and cluster CD95 or CD40, respectively (7–10).

Although the central role of receptor clustering for signaling is widely accepted and although many intracellular signaling mechanisms of the clustering process have been identified, the role of the ligand in the clustering of its cognate receptor is unknown. To test the hypothesis that clustering of a ligand is involved in the clustering of its receptor, we examined the CD40-CD40 ligand system.

CD40 ligand is an ~30-kDa type II membrane glycoprotein consisting of 261 amino acids (11). The molecule is primarily expressed in T lymphocytes, but it is also present in mast cells, basophils, NK cells, monocytes, B lymphocytes, and platelets (12). The interaction between the CD40 ligand and its cognate receptor, CD40, is one of the key events of B-cell activation and is required for Ig class switching (13, 14). Thus, B cells from patients who lack a functional CD40-CD40 ligand system are unable to switch from IgM to IgG production upon antigenic challenge (15, 16). These patients suffer from a severe immunodeficiency characterized by normal or elevated IgM levels, low IgG concentrations, and severe, recurrent bacterial infections (15, 16).

We and others have suggested that the CD40 ligand not only functions as a ligand for CD40 but is also involved in outside-to-inside signaling (17–21). This function might be immanent to the entire nerve growth factor/TNF ligand family, because outside-to-inside signaling has been recently also described for TNFα (22–24). Cellular activation via TNFα up-regulates the expression of interleukin-2, interferon-γ, and E-selectin and triggers an increase of intracellular free Ca2+ (22–24). Antibody-mediated stimulation of EL4 T cells via the CD40 ligand stimulates Jun N-terminal kinases, p38 kinase, Rac-1, the Src-like tyrosine kinase p56lck, and protein kinase C (17, 18). In addition, co-ligation of the CD40 ligand to the CD3 complex triggers an enhanced production of interleukin-10, interferon-γ, and TNFα and mediates apoptosis of T cells (19).

Here, we tested the hypothesis that signaling via the CD40 ligand is required to enable the CD40 ligand to deliver a potent signal to CD40. CD40 clustering requires a p53-dependent activation of the ASM, a formation of ceramide, and the formation of ceramide-enriched membrane domains, which trap and...
cluster CD40 ligand. Inhibition of CD40 ligand clustering prevents CD40 clustering, demonstrating that primary clustering of the CD40 ligand is essential in CD40-mediated activation of B lymphocytes.

MATERIALS AND METHODS

Cells and Stimulation—Human JY B cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA). CD40 ligand-positive murine EL4 cells were a kind gift from Dr. R. H. Zuberler (University Hospital, Geneva, Switzerland).

Syngeneic p53 knock-out mice and normal control mice were obtained from the Jackson Laboratory (Bar Harbor, ME), and ASMC-deficient and normal control mice were a kind gift from Dr. R. Kolesnick (Memorial Sloan-Kettering Cancer Center, New York).

Mature spleen cells were purified by centrifugation through a Ficol density gradient, and B cells were removed using anti-Ig-coated microbeads (Miltenyi, Bergisch-Gladbach, Germany). To induce CD40 ligand expression, we stimulated the remaining cells with plate-bound anti-TCR 145-2C11 (BD-PharMingen, San Diego, CA) for 9 h. Expression of CD40 ligand was confirmed by FACS analysis (BD, Franklin Lakes, NJ).

To overexpress ASMC, we transfected CD40 ligand-positive Jurkat cells with a previously described expression vector for the ASMC (pEF-ASM) containing a vector (pEF) (25). Cells were cloned using G418 resistance and reestablished from frozen stocks every 4 weeks. ASMC-deficient or normal control cells were obtained from spleens of ASMC knock-out or syngenic normal control mice.

All cells were maintained in phenol red-free RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (all from Invitrogen), and 50 μg/ml β-mercaptoethanol.

Anti-human CD40 (clone 5C3) and anti-murine CD40 ligand (clone MR1) were obtained from BD-PharMingen; anti-asm (clone 15B4) was obtained from Alexis Biochemicals (San Diego, CA); goat polyclonal anti-ASM antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Cells were stimulated with 1 μg/ml anti-CD40 ligand antibodies. Nystatin (10 μg/ml), filipin (0.5 μg/ml), and α- and β-methyl-cyclodextrins (1 mM each) (Sigma), respectively, were added to the cells 20 min before any stimulation. Cytochalasin D (5 μg/ml) was added 30 min prior to the stimulation. All stimulations were performed in a solution of 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, and 0.8 mM MgSO4 (H/S) supplemented with 0.5% fetal calf serum.

To investigate the function of CD40 ligand clustering in the process of CD40 clustering, we co-incubated CD40 ligand-positive EL4 cells with CD40-positive JY cells (cell ratio, 2:1) at 37 °C for the indicated time.

Fluorescence Microscopy—Lymphocytes were stimulated via CD40 or CD40 ligand, washed with ice-cold H/S supplemented with 1 mM ortho-phenanthroline to prevent CD40 ligand shedding, and incubated for 20 min in ice-cold H/S supplemented with 2% fetal calf serum, 0.01% Na3Vo4, and 1 mM ortho-phenanthroline to block sites of nonspecific binding. Fe receptors on B cells were blocked by an irrelevant rabbit IgG (20 μg/ml) during a 45-min incubation at 4 °C. Cells were washed and incubated for 45 min with 500 ng/ml anti-CD40 5C3, 1 μg/ml anti-murine CD40 ligand MR1, 1 μg/ml anti-CD40 ligand 15B4, or 5 μg/ml goat anti-ASM. All reagents were diluted in a solution of H/S, 1% fetal calf serum, 0.01% Na3Vo4, and 1 mM ortho-phenanthroline. Cells were washed and incubated for an additional 45 min with cyanine 3.18 (Cy3)- or cyanine 5.18 (Cy5)-labeled F(ab)2, fragments of anti-mouse, anti-goat, or anti-hamster antibodies (all from Jackson ImmunoResearch Laboratories, West Grove, PA), respectively. Double stainings were performed by consecutive staining with 1 μg/ml Cy3-labeled anti-murine CD40 ligand MR1 and 1 μg/ml Cy5-coupled anti-CD40 ligand 15B4 antibodies.

After staining, cells were fixed for 15 min in 1% (v/v) paraformaldehyde in phosphate-buffered saline and immobilized for 15 min on glass coverslips coated with 1% (v/v) poly-L-lysine.

Control experiments were performed with irrelevant monoclonal antibodies. No substantial amount of the control antibodies bound to the cells.

Cells were analyzed on a conventional Zeiss fluorescence microscope (Thornwood, NY) or a Leica TCS NT scanning confocal microscope (Bensheim, Germany). Clustering was defined as one or several intense spots of fluorescence on the cell surface; resting cells displayed a homogenous distribution of fluorescence throughout the membrane. In each experiment, the presence or absence of clustering in at least 200 randomly chosen cells was scored by two independent observers. In other control experiments, paraformaldehyde fixation was omitted, and capping was directly analyzed by fluorescence microscopy. The results from these controls were identical to those of paraformaldehyde-fixed samples, excluding the possibility that CD40 ligand clustering was an artifact caused by paraformaldehyde.

To test the specificity of the anti-ceramide antibody 15B4, we incubated JY cells with 1 unit of purified sphingomyelinase (Sigma) for 5 min or with 1 μM C16-ceramide (Biomol) for 5 min. Cells were then stained for surface ceramide as above.

Phase Display—To identify proteins associated with the intracellular domain of the CD40 ligand, we used a phage display method. The experiments were performed exactly as described in the protocol for the CLONTECH Easy Match Display Library (CLONTECH, Heidelberg, Germany). Glutathione S-transferase (GST) fusion proteins containing the intracellular domain of the CD40 ligand were expressed in and purified from Escherichia coli DH5a and immobilized in 96-well plates for 12 h at 4 °C. The plates were washed, blocked with 1% bovine serum albumin, and incubated for 20 h at 4 °C, and washed again. The immobilized GST proteins were then incubated for 60 min at 37 °C with 109 plaque-forming units of M13 phages expressing a peptide library that was generated from 3 × 106 independent clones of 0.3–3.0-kilobase pair length. The inserts were from a spleen cDNA library (CLONTECH). Phages expressing the peptide library were amplified in E. coli superinfected with the M13K07 helper phage. The plates were washed five times with phosphate-buffered saline and 0.05% Tween 20. Trapped phages were rescued by infection of added TG1 cells. Phages were enriched and used for a second round of panning on immobilized CD40 ligand. After two more rounds of panning on CD40 ligand, phagemid DNA was prepared, and the inserts were sequenced.

Immunoprecipitation Experiments—To show the association of CD40 ligand with p53 in vivo, co-immunoprecipitation experiments were performed. Cells were stimulated via CD40 ligand and lysed for 5 min on ice in a solution of 25 mM HEPES (pH 7.4), 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 100 mM NaCl, 10 mM NaF, 10 mM NaN3, 10 mM NaVO3, 10 mM EDTA, and 10 μg/ml each aprotinin and leupeptin (buffer A). Nuclei were pelleted by centrifugation at 14,000 rpm for 15 min at 4 °C, and the supernatants were subjected to anti-CD40 ligand or 5 μg per sample of monoclonal anti-p53 (Oncogene Science, Cambridge, MA) antibodies. Immunocomplexes were immobilized with 30 μl of protein (A/G)-coupled agarose and incubated for 60 min. Immunocomplexes were washed six times in buffer A and finally resuspended in SDS sample buffer (final concentration, 60 mM Tris (pH 6.8), 2.3% SDS, 10% glycerol, and 5% β-mercaptoethanol). The samples were boiled for 5 min at 95 °C, separated by SDS-PAGE, and transferred onto nitrocellulose membranes (Amersham Biosciences) and blocked by 4% bovine serum albumin in Tris-buffered saline-Tween. Blots containing CD40 ligand immunoprecipitates were incubated with 1 μg/ml anti-p53 antibodies for 2 h at 4 °C; blots from p53-immunoprecipitates were blotted with 1 μg/ml anti-CD40 ligand antibodies. The blots were extensively washed and incubated for 1 h with an antibody-conjugated alkaline phosphatase (Santa Cruz Inc.). Washed and bound proteins were detected by using the Tropix chemiluminescence system (Tropix Inc., Bedford, MA).

Co-precipitation of p53 with GST-CD40 Ligand Proteins—The intracellular domain of the CD40 ligand was subcloned into the pGEX-vector as above and expressed in E. coli DH5a after a 3-h treatment with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside. Bacteria were treated for 20 min with lysozyme and lysed in buffer A, and GST-CD40 ligand was purified using glutathione-Sepharose and five washes with buffer A. EL4 cells were lysed in buffer A and incubated for 4 h at 4 °C with 2 μg/ml GST-CD40 ligand. Samples were washed six times in buffer A, eluted in SDS-sample buffer, separated by 10% SDS-PAGE, transferred on nitrocellulose membranes, blotted with anti-p53 antibodies, and detected using the Tropix chemiluminescence system as above.

ASM Activity—After stimulation via the CD40 ligand, cells were lysed in ice-cold 50 mM Tris (pH 7.4), 10 mM bacitracin, 1 mM benzamidine, 1 mM NaN3, 10 μg/ml each aprotinin and leupeptin, 0.1 mg/ml soybean trypsin inhibitor, and 0.2% Triton X-100 and sonicated three times for 10 s each. Samples were centrifuged for 5 min at 600 × g, and the supernatants were supplemented with an equal volume of 50 mM

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CD40 Ligand Clustering
Tris (pH 7.4), 3% Nonidet P-40, 1% Triton X-100, 1 mM Na₃VO₄, and 10 μg/ml each aprotinin and leupeptin. ASM was immunoprecipitated with a goat polyclonal anti-ASM antibody (kindly provided by Prof. Dr. K. Sandhoff, University of Bonn, Germany) for 4 h and immobilized by agarose-coupled protein A/G (Santa Cruz Biotechnology). Immunoprecipitates were washed three times with 50 mM Tris (pH 7.4), 3% Nonidet P-40, 1% Triton X-100, 1 mM Na₃VO₄, and 10 μg/ml each aprotinin and leupeptin and then three times in a solution of 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, 1 mM Na₃VO₄, and 10 μg/ml each aprotinin and leupeptin. Immunoprecipitates were finally resuspended in 30 μl of [¹⁴C]sphingomyelin (100 nCi per sample; 54.5 mCi/mmol; PerkinElmer Life Sciences) in assay buffer, which contained 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA, and 0.05% Nonidet P-40. The substrate [¹⁴C]sphingomyelin was dried and solubilized by 10-min bath sonication in assay buffer. The enzyme reaction was performed for 30 min at 37°C and stopped by extraction in 800 μl of CHCl₃/CH₃OH (2:1, v/v) and 250 μl of H₂O. Degradation of [¹⁴C]sphingomyelin to ceramide and water-soluble [¹⁴C]phosphorylcholine by ASM was measured by liquid scintillation analysis of an aliquot taken from the upper phase.

Ceramide Formation—EL4 cells were labeled for 48 h with 2 μCi/ml [³H]serine (27 mCi/mmol; PerkinElmer Life Sciences), stimulated via the CD40 ligand, and extracted with CHCl₃, CH₃OH, H₂O, and pyridine (dilution ratio, 60:160:6:1). The lower phase was collected, dried, treated with methanolic NaOH at 37°C for 2 h to degrade phospholipids, and reextracted with CHCl₃, CH₃OH, and 1 M HCl (dilution ratio, 100:100:1). The lower phase was dried and resuspended in a solution of CHCl₃ and CH₃OH (dilution ratio, 95:5), and lipids were separated on silica G60 thin layer high performance thin layer chromatography plates (Merck) with CHCl₃, CH₃OH, and CaCl₂ (dilution ratio, 65:35:8). Endogenous ceramide was identified as that material that comigrated with a radioactive [¹⁴C]ceramide standard. Results obtained with [³H]serine labeling were confirmed by using the diacylglycerol kinase method (not shown) (25).

RESULTS

To investigate whether redistribution of a ligand in the cell membrane is required for the clustering of the cognate receptor, we used the CD40-CD40 ligand system. We investigated whether CD40 ligand clusters after incubation with an anti-CD40 ligand antibody or upon co-incubation with CD40-positive B lymphocytes. The results reveal a very rapid clustering of CD40 ligand in purified murine T lymphocytes (Fig. 1A) or EL4 cells (Fig. 1B), respectively, upon stimulation via the CD40 ligand (Fig. 1, A and B). Quantitative analysis shows that ~50% of all cells cluster CD40 ligand within 5 min (Fig. 1C). We have previously shown that ceramide released by the ASM mediates a reorganization of small lipid rafts to larger platforms and a trapping of, at least, some receptors in these platforms (7–10). To test the hypothesis that ASM and ceramide are involved in CD40 ligand clustering, we determined the surface translocation and enzymatic activity of the ASM and formation of ceramide upon stimulation of EL4 cells via CD40 ligand. The experiments reveal a very rapid and transient activation of the ASM and a concomitant formation of ceramide upon stimulation of the cells via CD40 ligand (Fig. 2A). Enzyme stimulation correlated with a translocation of the ASM onto the extracellular leaflet of the cell membrane (Fig. 2B). The notion that membrane platforms are important for CD40 ligand clustering is supported by the finding that CD40 ligand clusters in Cy5-choleratoxin-positive membrane platforms (Fig. 2C). Cell surface activity of the ASM correlated with a formation of extracellularly oriented ceramide (Fig. 2D) upon CD40 ligand stimulation. Fluorescence microscopy studies re-

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**Fig. 1. Stimulation of the CD40 ligand results in clustering of the molecule.** Rapid clustering of the CD40 ligand occurs in murine peripheral T cells (A) and in the T-cell line EL4 (B) after stimulation via the CD40 ligand or upon co-incubation with CD40-positive B cells. Displayed are typical results of fluorescence microscopy studies (A and B) and the quantitative analysis of CD40 ligand clustering (C). Cells were stimulated for the indicated time via CD40 ligand and stained with Cy5-labeled anti-CD40 ligand antibodies. Shown is the mean ± S.D. or representative results from five independent experiments each, respectively.
veal that surface ceramide also localized to larger platforms or cluster-like structures (Fig. 2E). Signaling platforms appear to be modified by ceramide, which co-localizes with CD40 ligand within the cluster structures (Fig. 2F). The confocal microscopy studies also indicate that the CD40 ligand and ceramide cluster consist of several small subunits forming a larger platform.

Fig. 2. CD40 ligand stimulates ASM-mediated ceramide formation and co-localizes with ceramide-enriched membrane domains. A, activation of EL4 cells via the CD40 ligand stimulates ASM and triggers a formation of ceramide within seconds. Shown is the mean ± S.D. of three independent experiments. Open circles, ASM activity; closed circles, ceramide formation. B, ASM stimulation correlates with a translocation of ASM onto the cell surface upon stimulation of EL4 cells via CD40 ligand. Surface ASM was detected by FACS analysis of cells incubated with Cy3-labeled goat anti-ASM. Shown is a representative FACS analysis from four independent experiments. C, CD40 ligand clusters in cholera toxin-positive, distinct domains of the cell membrane as demonstrated by confocal microscopy. The figure is representative of five independent experiments with very similar results. 100 cells per experiment were analyzed. D, activation and surface translocation of ASM correlates with a formation of extracellularly oriented ceramide. Cells were incubated with the Cy3-coupled anti-ceramide antibody 15B4 and analyzed by FACS. Displayed is a representative result from four independent studies. E, surface ceramide revealed a cluster-like distribution pattern and was localized into distinct membrane patches. Shown is a fluorescence microscopy analysis from five independent experiments with analysis of 100 cells each. F, clustered CD40 ligand co-localized with ceramide in the cell membrane. EL4 cells were stimulated via the CD40 ligand and labeled with Cy3-coupled anti-CD40 ligand and Cy5-labeled anti-ceramide 15B4. The overlays (Cy3/Cy5) indicate that CD40 ligand co-localizes with ceramide upon clustering. Shown are confocal images that are representative of four similar experiments with analysis of 100 each. G, FACS analysis reveals that unstimulated cells or cells lacking the ASM do not interact with the anti-ceramide 15B4 antibody. CD40 ligand stimulation of ASM-deficient cells (ASM<sup>−/−</sup>) also fails to result in a binding of the anti-ceramide antibody, whereas ASM-expressing cells (ASM<sup>+/+</sup>) rapidly bind the anti-ceramide antibody upon CD40 ligand stimulation. Experiments were repeated twice with very similar results. H, incubation of Jurkat cells with 1 μM C<sub>16</sub>-ceramide or 1 unit/ml sphingomyelinase for 5 min each results in binding of the anti-ceramide 15B4 antibody. Ceramide spontaneously forms platforms in the cell membrane. Cells were stained with Cy3-labeled anti-ceramide 15B4 antibodies and analyzed by fluorescence microscopy. Shown is a typical result of three independent experiments.
Several control experiments confirm the specificity of the anti-ceramide antibody; FACS analysis reveals that the anti-ceramide antibody did not significantly bind to unstimulated cells (Fig. 2, D and G) and thus excluded the possibility that the 15B4 antibody might react substantially with cholesterol, sphingomyelin, or other phospholipids in the cell membrane. Likewise, the antibody did not bind to ASM-deficient cells, even after stimulation via CD40 ligand (Fig. 2 G). Loading intact cells with C16-ceramide or treatment with purified sphingomyelinase conferred strong antibody binding to the cell surface (Fig. 2H), whereas untreated cells were negative. C16-ceramide or endogenous ceramide generated by purified sphingomyelinase spontaneously formed platforms (Fig. 2H).

These data indicate a co-localization of CD40 ligand, ASM, and ceramide in cluster-like structures.

Next, we tested the significance of the ASM for CD40 ligand clustering using ASM-deficient T cells obtained from ASM knock-out mice. The studies show that CD40 ligand clustering is absent in T cells of ASM-deficient mice, whereas rapid aggregation of the CD40 ligand occurs in T cells of ASM-expressing control mice upon stimulation (Fig. 3A).

Sphingolipid-enriched rafts contain cholesterol, which appears to function as a spacer between the bulky sphingolipids; therefore, the extraction of cholesterol from cells destroys sphingolipid-rich rafts (26). To further study the role of sphingolipid-rich rafts in CD40 ligand clustering, we incubated EL4 cells with \( \beta \)-cyclodextrin, nystatin, and filipin, agents that interfere with the metabolism of cellular cholesterol and destroy sphingolipid-enriched rafts. Treatment with these reagents prevented the clustering of CD40 ligand (Fig. 3B), whereas treatment with \( \alpha \)-cyclodextrin, an inactive stereoisomer of \( \beta \)-cyclodextrin, had no effect (Fig. 3B).

These results indicate that the initial contact between CD40 and the CD40 ligand causes ASM to translocate, become activated, and mediate clustering of the CD40 ligand in ceramide-enriched rafts.
To identify intermediates of the signaling pathway from CD40 ligand to ASM, we searched for proteins associating with the CD40 ligand. To this end, we performed a phage display analysis and used the intracellular part of the CD40 ligand as bait to screen a phage peptide expression library. These screens, which were confirmed by results of screening a second, independent peptide library, revealed the association of peptides with the CD40 ligand, which were identical or highly homologous to sequences in p53 (Fig. 4A). To further investigate the association of p53 with CD40 ligand, we tested whether p53 binds to a purified GST-CD40 ligand fusion protein. To this end, GST-CD40 ligand fusion proteins were incubated with lysates from EL4 cells, and binding proteins were eluted and analyzed by Western blotting using anti-p53 antibodies. The results confirm the phage display data and reveal an association of p53 with GST-CD40 ligand (Fig. 4B).

To show an in vivo association between p53 and CD40 ligand, we performed co-immunoprecipitation experiments by using anti-p53 antibodies to determine whether p53 was present in immunoprecipitates of CD40 ligand and by using anti-CD40 ligand antibodies to determine whether CD40 ligand was present in immunoprecipitates of p53. These studies revealed an in...
CD40 ligand clustering

Fig. 4. CD40 ligand associates with p53. A–C, phage display analysis and co-immunoprecipitation experiments reveal an association of the CD40 ligand with p53. A, shown are peptide sequences homologous to p53 obtained by phage display analysis using GST-CD40 ligand fusion protein as bait. Results were confirmed by a second phage analysis using an independent library. Identical amino acids in p53 and the peptides are indicated by a box. B, GST-CD40 ligand fusion proteins bind to p53. Lysates from EL4 cells were incubated with GST-CD40 ligand fusion proteins or GST that were immobilized to glutathione-Sepharose. The samples were washed, blotted, and tested for the presence of p53 using an anti-p53 antibody and the Tropix detection system. The data were reproduced four times. C, co-immunoprecipitation experiments of p53 and CD40 ligand from peripheral blood T lymphocytes demonstrate the in vivo association between p53 and CD40 ligand. The upper blots show the presence of p53 in CD40 ligand immunoprecipitates or vice versa. The lower Western blots were performed with the same antibody as used for immunoprecipitation to indicate similar amounts of CD40 ligand or p53, respectively, in all specific immunoprecipitates. Controls for CD40 ligand immunoprecipitates (left blot) were performed with an isotype-matched irrelevant antibody for immunoprecipitation. Controls for p53 immunoprecipitates (right blot) were performed with anti-p53 antibodies on lymphocytes from p53-deficient mice, which confirms the specificity of the p53-CD40 ligand association. Heavy chain indicates the heavy chain of the antibody used for immunoprecipitation. Representative results from three independent experiments are presented.

vivo association between the CD40 ligand and p53 (Fig. 4C). Experiments using p53 knock-out cells confirmed the specificity of the co-immunoprecipitation experiments.

Next, we tested the function of the association of CD40 ligand with p53 by measuring the translocation and activation of ASM and the clustering of the CD40 ligand in peripheral T lymphocytes from normal or p53 knock-out mice. While normal cells rapidly responded to stimulation via CD40 ligand by translocating ASM to the cell surface (Fig. 5A), activation of the ASM (Fig. 5B), formation of ceramide (Fig. 5B), and clustering CD40 ligand (Fig. 5, C and D), p53-deficient cells failed to respond (Fig. 5, A–D). These findings indicate a central role of p53 in CD40 ligand-mediated activation of the ASM and, finally, clustering. To further characterize the association of p53 with CD40 ligand, we tested the role of the ASM and cytoskeleton elements for the association of the two proteins. The data reveal that ASM itself did not influence the association, and a very similar association of CD40 ligand with p53 was observed in normal control cells, cells lacking the ASM, or cells overexpressing the ASM —5 fold (Fig. 5E). Likewise, pretreatment of the cells with cytochalasin D or nocodazole that blocks the assembly of microtubules or disrupts actin filaments, respectively, did not alter the association of the two proteins (Fig. 5F), suggesting that the association of p53 with CD40 ligand does...
FIG. 5. **p53 is required for ASM translocation, activation, and CD40 ligand clustering.** A and B, T cells obtained from p53-deficient mice failed to respond to stimulation via CD40 ligand. Unlike wild-type cells, p53-deficient cells do not show surface translocation (A) or activation (B) of ASM and formation of ceramide (B) in response to stimulation via CD40 ligand. A representative FACS analysis from three similar experiments and the mean ± S.D. of ASM activity or ceramide formation, respectively, measured in three independent experiments are shown. **Circles,** ASM activity or ceramide formation in control cells; **squares,** ASM activity or ceramide formation in p53-deficient cells. **Open symbols,** ASM activity; **closed symbols,** ceramide formation. C and D, genetic deficiency of p53 prevents clustering of the CD40 ligand. Peripheral T cells from p53-deficient or wild-type mice were stimulated via the CD40 ligand, and clustering of the CD40 ligand was evaluated by fluorescence microscopy (C). Shown are a representative result (C) and the mean ± S.D. of the quantitative analysis (D) of four independent experiments in which 100 cells per sample were analyzed. E, deficiency or overexpression of the ASM does not alter the association of the CD40 ligand with p53. Murine ASM-deficient T cells,
FIG. 6. Clustering of CD40 ligand is required for the clustering of its cognate receptor, CD40. A and B, co-incubation of CD40 ligand-positive EL4 T cells with JY B cells results in clustering of CD40 on JY B cells (A). Cells were stained with Cy3-coupled anti-CD40 5C3 antibodies. No cross-reaction with the T cells was observed. Displayed is a representative fluorescence microphotograph from four independent experiments analyzing a total of 400 cells. B, genetic deficiency of ASM or p53 in peripheral blood T cells prevents clustering of CD40. T cells from wild-type control mice rapidly trigger clustering of CD40 on co-incubated JY B cells. Given are the mean ± S.D. from four independent experiments with analysis of 100 cells each. Peripheral T cells were obtained from \( h_{11002} \), \( p53^-/- \), or wild-type control mice, respectively. These cells were stimulated for 8 h with the anti-CD3 antibody 145-2C11, subjected to FACS analysis to test for expression of CD40 ligand, and co-incubated with CD40-expressing JY cells. CD40 clustering on JY cells was determined upon staining with the anti-CD40 antibody 5C3. EL4 cells did not react with the anti-CD40 antibody 5C3. C, likewise, preincubation of EL4 cells with \( \beta \)-cyclodextrin, nystatin, or filipin to disrupt sphingolipid-enriched rafts destroys their ability to stimulate clustering of CD40 on co-incubated JY B cells. The inactive stereoisomer \( \alpha \)-cyclodextrin does not affect CD40 clustering elicited by co-incubation with CD40 ligand-positive EL4 cells. Shown is the mean ± S.D. of three independent experiments each. D, the summary suggests that stimulation via CD40 ligand triggers a surface translocation and activation of the ASM and a release of ceramide, which mediates the fusion of small rafts to large platforms. Those platforms serve to cluster CD40 ligand, which functions as a prerequisite for CD40 clustering and, finally, CD40-dependent signaling in B lymphocytes.

normal murine control T cells, Jurkat cells overexpressing the ASM, and control transfected Jurkat cells (each \( 20 \times 10^6 \) cell equivalents/lane) show very similar levels of p53-CD40 ligand association. Shown is a representative blot of three independent experiments. F, pretreatment of EL4 cells with cytochalasin D or nocodazole to disrupt the actin cytoskeleton or to prevent microtubuli polymerization, respectively, does not significantly alter the association of CD40 ligand with p53. Displayed is a typical result of three independent experiments.
not depend on microtubules or the actin cytoskeleton, respectively.

Next, we studied the significance of CD40 ligand clustering for the formation of the synapse. Cytoskeletal disruption with cytochalasin D, nocodazole, or filipin led to major defects in the formation of a functional immune synapse with CD40 (20, 44, 45). Thus, clustering of CD40 ligand might be required only for the initiation of CD40 clustering and not for the transmission of a sustained signal via clustered CD40.

Surface ceramide was detected by the anti-ceramide antibody 15B4, which was raised against C16-ceramide coupled to bovine serum albumin. Our data indicate that the antibody specifically detects ceramide and does not cross-react with cholesterol, sphingomyelin, or other membrane components that are present in unstimulated or ASM-deficient cells. However, we cannot exclude the possibility that metabolites of ceramide (i.e., glycospedal ceramide derivatives or sphingosine species) are detected by the antibody and have effects on membrane reorganization that are similar to those of ceramide.

In summary, our results demonstrate a novel role of ligand clustering in the process of receptor clustering. We showed that upon initial contact with the CD40 receptor, the CD40 ligand actively and rapidly clusters in or at ceramide-enriched signaling platforms. Clustering of CD40 ligand appears to be required for subsequent CD40 clustering, which has been shown to be central for the initiation of CD40 signaling (10, 44, 45). Thus, clustering of CD40 ligand plays an essential role in the formation of a functional immune synapse with CD40.

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