CD95 Signaling via Ceramide-rich Membrane Rafts*

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Clustering seems to be employed by many receptors for transmembrane signaling. Here, we show that acid sphingomyelinase (ASM)-released ceramide is essential for clustering of CD95. In vitro and in vivo, extracellularly orientated ceramide, released upon CD95-triggered translocation of ASM to the plasma membrane outer surface, enabled clustering of CD95 in sphingolipid-rich membrane rafts and apoptosis induction. Whereas ASM deficiency, destruction of rafts, or neutralization of surface ceramide prevented CD95 clustering and apoptosis, natural ceramide only rescued ASM-deficient cells. The data suggest CD95-mediated clustering by ceramide is prerequisite for signaling and death.

Stimulation of a variety of surface receptors including the T-cell receptor/CD3 complex (1, 2), B-cell receptor (3), tumor necrosis factor receptor (TNF-R) (4), CD2, CD44, L-selectin, or integrins (5) results in clustering of these receptors, which appears to be required for rapid and efficient receptor-mediated signaling. Recent studies on peptide antigen-induced signaling via the T-cell receptor/CD3 complex indicated that receptor aggregation rather than conformational changes of the intracellular part of the T-cell receptor/CD3 complex upon ligand binding is the predominant mechanism mediating signal transmission (1). Evidence suggests that many receptors aggregate in distinct cholesterol- and sphingolipid-rich membrane microdomains or rafts (6–8). This notion is supported by the finding that disruption of rafts prevents clustering of many receptors including the B-cell receptor (9), CD45 (10), Fcy (11), or the TNF-R (12). Rafts seem to exist as preformed entities in the membrane of resting cells (13); whether receptor stimulation induces a biologically relevant modification of these rafts is unknown. Likewise, mechanisms mediating the trapping of activated receptor molecules within rafts require definition.

In the present studies, we have investigated whether a hydrolysis of sphingomyelin to ceramide and, thus, a change in the composition of rafts contributes to clustering of receptor molecules. Ceramide is released by the activity of at least three forms of sphingomyelinases with an acidic, neutral, or basic pH optimum (14). Of these enzymes only the acid sphingomyel-

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MATERIALS AND METHODS

Cells and Stimulation—Human ASM- or acid ceramidase (AC)-deficient lymphocytes or fibroblasts, respectively, were obtained from patients with Niemann Pick disease type A (NDPA) or Farber disease. JY and Jurkat were from ATCC. All lymphocytes were grown in phenol red-free RPMI 1640 supplemented with 10% fetal calf serum, 10 mM HEPES, pH 7.4, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 μM non-essential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin (all Life Technologies, Inc.) and 50 μg/ml β-mercaptoethanol. Fibroblasts and LN229 gloma cells (kindly provided by Dr. M. Weller) were cultured in phenol red-free DMEM supplemented as above. Anti-human CD95 antibodies were from UBI, anti-human CD95 ligand NOK-1 from PharMingen, anti-keramide 15B4, recombinant CD95 ligand, and anti-FLAG antibodies from Alexis. Anti-CD40 5C3, anti-L-selectin DREG 56 and anti-integrin 6.7 were from PharMingen, anti-VSV P5D4 from Roche Molecular Biochemicals. Reconstitution of NPDA or Darber B cells for ASM or AC, respectively, was by electroporation using ASM and AC constructs subcloned into the pJK or pEF vectors (pJK-asm, pEF-asm), which regulate gene expression under control of an elongation factor promoter. In addition, JY B cells were transfected with wild-type ASM or ASM fused with tag sequences of VSV and Myc (pJKVSV-asm, pEF-Myc-asm). Empty vec-

tors (pJK, pEF) were used as controls. The pJK vector also encodes a single chain antibody fused with a Myc tag permitting isolation of transfected cells by panning with anti-Myc-9E10 (Roche Molecular Biochemicals) as described (23). Expression of ASM constructs was confirmed by FACS, and measurement of ASM activity revealed a 3–5-fold overexpression.

Stimulation with anti-CD95 CH11 was performed at 30 ng/ml if not otherwise indicated. CD95 ligand was added to cells at 20 ng/ml for 10 min at 4 °C. Cells were washed, and cellular activation was initiated by addition of 100 ng/ml anti-FLAG F(ab)2 antibodies at 37 °C. Cells were stimulated for 2 min for capping experiments and 4 h for apoptosis studies, unless otherwise indicated.

Nystatin was used at 10 μg/ml, filipin at 0.5 μg/ml, α and β-cyclo-
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dextrins at 1 mM each. They were added 30 or 15 min prior to infection or anti-CD95 stimulation, respectively. The anti-ceramide antibody 15B4 was used at 1 µg/ml. C16-ceramide, C20:4 arachidonic acid, sphingomyelin, dihydro-C2- ceramide, and dihydro-C16-ceramide were used at 1 µM each in octyl-glucopyranoside.

For fluorescence microscopy, lymphocytes were immobilized on glass coverslips coated with 1% (v/v) poly-L-lysine for 15 min, stimulated or left unstimulated, fixed for 15 min in 1% paraformaldehyde (PFA) (v/v) in PBS (PFA/PBS), and washed in 0.05% Tween 20/PBS and 0.1% bovine serum albumin/C-PBS (v/v, Aurion EM Reagents). Where indicated cells were permeabilized by a 10-min incubation in 0.1% Triton X-100/PBS. Cells were stained for 45 min each with a polyclonal antibody for a monoclonal mouse anti-ASM (23) followed by incubation with 0.5 µg/ml Texas Red (TR)-conjugated F(ab)2-fragments of anti-goat or mouse antibodies (PharMingen), respectively. Cells were washed and stained for 45 min with 200 ng/ml FITC-labeled anti-CD95 CH11. Control stainings were performed with irrelevant monoclonal mouse antibodies or pre-bled goat antiserum. FC receptors on B-cells were blocked with a 45-min incubation with 20 µg/ml of an irrelevant rabbit antibody. All other antibodies did not bind to human Fc receptors. Staining was viewed using a conventional Zeiss fluorescence microscope or a Leica TCS NT scanning confocal microscope.

Cells for scanning electron microscopy were immobilized as above on plastic coverslips, stimulated, fixed in 4% PFA/PBS, blocked with a 15-min incubation of 1% BSA/C-PBS and incubated in this buffer for 15 min each with monoclonal anti-CD95 and a Nanogold-coupled goat anti-mouse antibody (NanoProbes, NY). Samples were postfixed in 4% PFA/PBS, dehydrated, critical-point-dried from CO2, sputter-coated with 1 nm Cr and examined at 10 kV accelerating voltage in a Hitachi S-800 field emission scanning electron microscope equipped with a detector for backscattered electrons of the YAG type. Magnification was ×30,000.

Lipid Studies—Binding of ceramide to intact cells was determined with a 2-min incubation with [3H]ceramide (1 µCi/ml) dissolved in 0.01% octyl-glucopyranoside. Cells were then extracted with CHCl3/CH3OH/H2O/pyridine (60:160:6:1) and lipids were separated by silica gel chromatography (24). The images were processed and digitized using Axon Imaging Systems software. Clustering was defined in all assays as one or several intense spots on the cell surface, whereas on resting cells, fluorescence was distributed homogenously throughout the membrane. In each experiment, 100–200 cells were scored for clustering by a blinded observer and confirmed by a second independent observer.

Apoptosis—Apoptosis of lymphocytes was measured as previously described (16) by binding of Fluos-labeled annexin V (Roche Molecular Biochemicals). Apoptosis was confirmed by DNA fragmentation using 10 µg/ml [3H]thymidine (8.3 Ci/mmol, PerkinElmer Life Sciences) labeling of the mammalian cells. To this end, cells were disrupted by one cycle of freezing and thawing, and unfragmented DNA was collected by filtration through glass fiber filters (Amersham Pharmacia Biotech) and counted by liquid scintillation spectrometry. Finally an aliquot of DNA incubation was treated with proteinase K, incubated one cycle of freezing and thawing, and unfragmented DNA was collected to determine radioactivity and ASM activity.

RESULTS

ASM and Ceramide Mediate CD95 Clustering in Sphingolipid-rich Rafts—Initially, we investigated whether stimulation via CD95 results in receptor clustering. To mimick the physiological interaction of membrane-bound CD95 ligand with CD95, Jurkat T or JY B lymphocytes were co-incubated with CD95 ligand-positive LN229 glioma cells. Stimulation via CD95 rapidly triggered CD95 clustering in the lymphocytes (Fig. 1A). Neutralization of tumor-associated CD95 ligand with a soluble recombinant Fc-CD95 protein completely abrogated clustering providing evidence for specificity of the receptor-ligand interaction. CD95 clustering was quantitatively comparable with that after stimulation with 30 ng/ml of anti-CD95 CH11 or 20 ng/ml cross-linked recombinant CD95 ligand (Fig. 1A). These doses were used in all subsequent experiments. Fig. 1B shows that clustering occurred within seconds after stimulation with anti-CD95 CH11 (Fig. 1B). Further, clustering was preceded by the primary formation of several small CD95 patches, which appeared to fuse to a large cluster/cap.

To gain insight into the molecular mechanism of CD95 clustering, we investigated whether ASM and ceramide might be involved. Whereas stimulation of ASM-retransfected lymphocytes with the stimulatory anti-CD95 CH11 or CD95 ligand resulted in rapid CD95 clustering (Fig. 1C), ASM deficiency abrogated CD95 clustering. Deficiency of ASM did not result in a general defect of receptor clustering, as capping of L-selectin or β2-integrin, which do not activate ASM (25), was unaltered in ASM-deficient cells (Fig. 1C). Genetic deficiency of AC strongly enhanced CD95 clustering (Fig. 1D). Because AC metabolizes ceramide and should function as a negative regulator of ceramide-mediated clustering by reducing cellular ceramide, these data support a pivotal role of ceramide for clustering.

To examine the role of ASM/ceramide in clustering of surface receptors, we determined the subcellular localization of ASM prior or after CD95 stimulation. Confocal microscopy on permeabilized (Fig. 2A) and intact cells (Fig. 2B), scanning electron microscopy (Fig. 2C), and FACS studies (Fig. 2D) revealed that ASM translocates onto the outer leaflet of the cell membrane and co-localizes with clustered CD95 in cholesterol- and sphingolipid-rich rafts upon CD95 stimulation. A small portion of ASM was located outside the cluster, a finding consistent with the primary formation of small microdomains that then fuse to a large cluster, as depicted in Fig. 1B. Consistent with an extracellular orientation of ASM, we detected binding of a monoclonal anti-ceramide antibody (15B4) to intact cells by FACS or fluorescence microscopy upon stimulation with anti-CD95 CH11 (Fig. 2E), whereas resting cells were essentially unreactive. These studies further revealed that ceramide was concentrated in distinct membrane domains of activated cells reminiscent of CD95- and ASM-containing clusters (Fig. 2E).

There is precedent for extracellular phospholipase action as PLA2 also translocates to the outer plasma membrane to release arachidonic acid from surface phospholipid (26, 27).

The role of sphingolipid rafts in CD95 clustering was further investigated by disruption of cellular cholesterol metabolism using filipin, nystatin, or β-cyclohexim. Loss of cholesterol destroys sphingolipid-enriched rafts. All three drugs, but not the inactive stereoisomer α-cyclohexim, abrogated CD95 clustering (Fig. 3A). Further, neutralization of surface ceramide by anti-ceramide 15B4, but not an irrelevant IgM, also abrogated CD95 clustering (Fig. 3B). Finally, addition of C16-ceramide (1 µM) to ASM-deficient B lymphocytes restored CD95 clustering whereas addition of C16-ceramide to normal cells was without effect (Fig. 3C). Incubation of cells with anti-ceramide antibody 15B4 abrogated the effect of C16-ceramide on CD95 clustering. C16-ceramide was incorporated into the same distinct membrane domains targeted by ASM upon stimulation as evidenced by co-localization of [3H]ceramide and ASM activity after subcellular fractionation across a sucrose density gradient (Fig. 3D). These studies
FIG. 1. CD95 clusters upon stimulation. A, co-incubation (10 min) of Jurkat T or JY B lymphocytes with LN229 glioma cells or stimulation for 2 min via CD95 using anti-CD95 CH11 or cross-linked CD95 ligand rapidly results in CD95 clustering. The specificity of the clustering process is evident as stimulation via CD95 does not result in clustering of other receptors including CD40, the B-cell receptor, L-selectin, or β2-integrin. Shown is the percentage of cells displaying CD95 clusters. The panel displays the mean ± S.D. of 30 experiments with analysis of a total of 3000 cells. B, a time course of clustering reveals very rapid clustering of CD95 with the primary formation of small rafts, which then seem to fuse to a large cap. Shown is a typical video image. The appearance of the red color in the video image reflects the increased intensity of the signal because of a higher concentration of CD95. The discrimination between red (capping) and green (not capping) enables an exact quantification of clustering. Shown is a typical result from 30 experiments. C, ASM deficiency prevents CD95 clustering upon stimulation. D, vice versa AC deficiency enhances CD95 clustering (n = 5 each; mean ± S.D.). Clustering of L-selectin or β2-integrin after specific receptor stimulation was not affected in ASM-deficient NPDA cells excluding a general clustering defect in these cells.
Acid Sphingomyelinase Mediates Receptor Clustering

A

- Unstimulated
- Stimulated via CD95

FITC-anti-CD95  Texas Red-anti-ASM  FITC-anti-CD95 + Texas Red-anti-ASM

B

- Unstimulated
- Stimulated via CD95

FITC-Choleratoxin  Cy3-anti-CD95  Cy5-anti-ASM  FITC/Cy3/Cy5

C

- Unstimulated
- Stimulation via CD95
suggest that ceramide within rafts is required for capping.

Ceramide-mediated Clustering Is Important for CD95-induced Apoptosis—Our studies showing that extracellularly orientated ceramide triggers CD95 clustering in rafts, raise the question of the significance of clustering for CD95-induced apoptosis. We therefore determined apoptosis via CD95 under conditions that block receptor clustering. We observed that genetic deficiency of ASM prevented anti-CD95 CH11-triggered apoptosis, an event restored by addition of natural C16-ceramide (Fig. 4A). Likewise, neutralization of surface ceramide using the anti-ceramide antibody 15B4 or destruction of lipid rafts by β-cyclodextrin, filipin, or nystatin inhibited CD95-induced apoptosis (Fig. 4B).

To confirm the significance of CD95 clustering for apoptosis in an in vivo situation, we employed the induction of apoptosis in mammalian cells upon infection with Pseudomonas aeruginosa (24); infection with P. aeruginosa induces apoptosis by up-regulation of endogenous CD95 and CD95 ligand on the cell surface resulting in activation of CD95 by its ligand (24). Genetic studies confirmed the strict requirement of the CD95/CD95 ligand system for P. aeruginosa-triggered cell death (24). Thus, this system enabled us to study the function of ASM and ceramide for CD95-triggered death without exogenous manipulation of the receptor/ligand system. The results show that ASM-deficient NPDA fibroblasts were resistant to P. aeruginosa-triggered apoptosis, whereas ASM-retransfected NPDA

**FIG. 2.** CD95 induces translocation of ASM onto the cell surface and release of extracellularly orientated ceramide. Confocal (A and B) and scanning electron microscopy (C) of JY cells revealed a translocation of the ASM onto the surface of the cell membrane and a co-localization of ASM with clustered CD95 in rafts. Rafts are indicated by FITC-labeled cholera toxin, which binds GM1 gangliosides enriched in rafts (34). In A, the cells were permeabilized; in B, the cells were left intact and thus do not show significant staining of (intracellular) ASM in the unstimulated sample. Because the GM1-binding β-subunit of cholera toxin forms a pore (35), some intracellular staining was observed for the toxin. In the scanning electron microscopy samples, the ASM was visualized by gold-coupled anti-ASM appearing on the cell surface as white dots. Shown is a representative result of 10 (A and B) or 3 (C) similar independent experiments. In A and B, a total of ~500 cells each were analyzed. D, transfection of Myc- or VSV-tagged ASM constructs confirms the translocation of the ASM onto the cell surface upon CD95 stimulation. JY B cells were stably transfected with e-Myc- or VSV-tagged ASM (pJK/myc-asm or pJK/vsv-asm) or the control vector (pJK) stimulated with anti-CD95 CH11 for 2 min, stained with FITC-labeled anti-Myc 9E10, anti-VSV P5D4, or goat anti-ASM and subjected to FACS analysis. The wild-type ASM- or pJK-transfected JY B cells served as controls. E, ceramide is exposed on the cell surface and localizes into distinct domains on the extracellular leaflet of the cell membrane upon stimulation via CD95. Shown is a representative FACS analysis and a typical video fluorescence image of five independent experiments each. Cells were stained with FITC-labeled anti-ceramide 15B4. The video imaging experiments analyzed a total of 500 cells. Please note that the sensitivity of the camera for unstimulated cells was 100 times higher than for the stimulated cells in order to detect a signal in resting cells.
fibroblasts readily died (Fig. 4C). Up-regulation of CD95 and CD95 ligand on the cells was not affected by expression of ASM (data not shown). The resistance of ASM-deficient cells indicates a role of the ASM for CD95-triggered apoptosis.

To define the role of CD95 clustering itself for apoptosis, we tested whether artificial cross-linking of CD95 restores apoptosis in $\text{asm}^{-/-}$ cells after infection with $P$. aeruginosa. If artificial cross-linking of CD95 overcomes apoptosis resistance in $\text{asm}^{-/-}$ cells, clustering can be considered pivotal for CD95 signaling. To this end, ASM-deficient NPDA fibroblasts were treated with aggregates of the non-stimulatory anti-CD95 antibody ZB4 during infection with $P$. aeruginosa. Anti-CD95 ZB4 binds but ordinarily does not activate CD95 or induce apoptosis. This antibody does not interfere with binding of CD95 ligand but blocks binding of anti-CD95 CH11. Therefore, it can be used as a tool to study the biological relevance of CD95 clustering; cross-linking of CD95 per se. D, C16-ceramide is incorporated into the same distinct membrane domains targeted by ASM upon stimulation. This is evidenced in experiments determining $[^{14}C16]$ceramide incorporation and ASM activity after subcellular fractionation of CD95-stimulated cells across a sucrose density gradient. Squares represent ASM activity; circles, radioactive ceramide.

**Fig. 3.** Surface ASM and ceramide mediate CD95 clustering. A and B, destruction of cholesterol- and sphingolipid-rich rafts (A) or neutralization of surface ceramide by binding of anti-ceramide 15B4 specifically prevented CD95 clustering (B). Clustering was analyzed by video-fluorescence microscopy. Shown is the mean ± S.D. of three independent experiments each. C, addition of natural C$_{16}$-ceramide (1 μM) restores CD95 clustering in ASM-deficient cells upon stimulation with anti-CD95 CH11. C$_{16}$-ceramide was added immediately prior to application of anti-CD95 CH11. Neither the solvent 0.01% octyl-glucopyranoside nor arachidonic acid (AA), sphingomyelin (SM), dihydro-C$_2$-ceramide, or dihydro-C$_{16}$-ceramide restored CD95 clustering in anti-CD95 CH11-treated NPDA cells. None of the reagents including C$_{16}$-ceramide triggered any CD95 clustering per se. D, C$_{16}$-ceramide is incorporated into the same distinct membrane domains targeted by ASM upon stimulation. This is evidenced in experiments determining $[^{14}C16]$ceramide incorporation and ASM activity after subcellular fractionation of CD95-stimulated cells across a sucrose density gradient. Squares represent ASM activity; circles, radioactive ceramide.
the mean upon infection. Monomeric anti-CD95 ZB4 was without effect. Shown is multimeric anti-CD95 ZB4 restored apoptosis in ASM-deficient cells. Blasts died by apoptosis. Artificial cross-linking of CD95 by addition of P. aeruginosa ASM abrogated apoptosis in fibroblasts infected for 30 min with the strain ATCC 27853 or 762, whereas ASM positive fibroblasts died by apoptosis (28) and by our preliminary data showing a 50–100-fold reduction of caspase 8 activation in cells lacking ASM upon CD95 stimulation, an event restored by ASM transfection. Translocated ASM, now in the proximity of sphingomyelin that resides in the outer surface of mammalian membranes, will release ceramide. In model membrane systems, ceramide has the unique capacity to spontaneously self-aggregate into microdomains (29–31). If this event occurred in a preformed microdomain such as caveolae or caveolar-like domains, re-ordering of this domain into a signaling structure might ensue. In fact, a number of groups have now published data demonstrating that ASM functions in sphingolipid-enriched microdomains in various cell types (21, 22). In addition, in model membranes ceramide-rich microdomains tend to fuse (32), and thus, ceramide might further trigger the fusion of rafts to larger platforms. This notion is supported by unpublished findings from our group that CD95 stimulation of NPDA cells expressing an inactive mutant of ASM induces translocation of this mutant protein onto multiple small spots on the cell surface, which fail to fuse into a large cluster. These platforms may finally interact with activated CD95 at the synapse between CD95 ligand and CD95, greatly increasing the stability of their interaction by (a) trapping and locking CD95 molecules within rafts, thus increasing the time of interaction with the ligand, (b) recruiting intracellular signaling molecules to CD95, (c) excluding inhibitory pathways, and/or (d) directly altering the affinity/avidity of CD95 for its ligand. As a result of receptor clustering, intracellular effector molecules mediating CD95 action are brought into close proximity, enabling their trans-activation. Thus, e.g., CD95 clustering may concentrate caspase 8 molecules within a confined area amplifying local proteolysis, even if primarily only a few dispersed caspase 8 molecules were active. In accordance with that model, high overexpression of the TNF-R results in its spontaneous multimerization and apoptosis of the transfected cells (33).

In the present study, we employed two anti-ASM antibodies, a polyclonal goat and a monoclonal mouse antibody. Both antibodies were raised against the full-length human ASM protein. To assure that ASM and not a cross-reacting protein was being recognized at the cell surface upon CD95 ligation, we repeated our studies using cells transfected with a Myc- or VSV-tagged ASM construct (see Fig. 2D). The pattern of detection of transfected ASM with either anti-Myc 9E10 or anti-VSV PS5D4 antibodies was identical to that detected using the anti-ASM antibodies. Further, lymphocytes derived from ASM knockout mice stained with either the goat or mouse anti-ASM antibodies failed upon P. aeruginosa infection, providing evidence that ASM-mediated clustering of CD95 is required for apoptosis induction.

**DISCUSSION**

Receptor clustering appears to be critical for transmembrane signaling through many receptors. Our results provide insight into molecular mechanisms of the clustering process and indicate a novel mechanism for receptor clustering. The data identify ASM, translocated onto the surface of sphingolipid-rich rafts, as a key player in CD95 clustering and signaling. The experiments using P. aeruginosa-induced up-regulation of CD95 and CD95 ligand indicate the significance of ASM/ceramide-mediated clustering of CD95 for apoptosis even under (patho)physiological conditions. Our data suggest the following function of ASM/ceramide for CD95 clustering: Interaction of membrane-bound CD95 ligand with CD95 results in a transient and weak activation of a limited number of CD95 trimers or oligomers insufficient to trigger apoptosis but sufficient for ASM translocation to the outer membrane leaflet. The notion of a primary weak activation of CD95 is supported by the findings that mere trimerization of CD95 by its ligand is insufficient to induce apoptosis (28) and by our preliminary data showing a 50–100-fold reduction of caspase 8 activation in cells lacking ASM upon CD95 stimulation, an event restored by ASM transfection. Translocated ASM, now in the proximity of sphingomyelin that resides in the outer surface of mammalian membranes, will release ceramide. In model membrane systems, ceramide has the unique capacity to spontaneously self-aggregate into microdomains (29–31). If this event occurred in a preformed microdomain such as caveolae or caveolar-like domains, re-ordering of this domain into a signaling structure might ensue. In fact, a number of groups have now published data demonstrating that ASM functions in sphingolipid-enriched microdomains in various cell types (21, 22). In addition, in model membranes ceramide-rich microdomains tend to fuse (32), and thus, ceramide might further trigger the fusion of rafts to larger platforms. This notion is supported by unpublished findings from our group that CD95 stimulation of NPDA cells expressing an inactive mutant of ASM induces translocation of this mutant protein onto multiple small spots on the cell surface, which fail to fuse into a large cluster. These platforms may finally interact with activated CD95 at the synapse between CD95 ligand and CD95, greatly increasing the stability of their interaction by (a) trapping and locking CD95 molecules within rafts, thus increasing the time of interaction with the ligand, (b) recruiting intracellular signaling molecules to CD95, (c) excluding inhibitory pathways, and/or (d) directly altering the affinity/avidity of CD95 for its ligand. As a result of receptor clustering, intracellular effector molecules mediating CD95 action are brought into close proximity, enabling their trans-activation. Thus, e.g., CD95 clustering may concentrate caspase 8 molecules within a confined area amplifying local proteolysis, even if primarily only a few dispersed caspase 8 molecules were active. In accordance with that model, high overexpression of the TNF-R results in its spontaneous multimerization and apoptosis of the transfected cells (33).

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to show a signal, supporting the specificity of these antibodies for ASM.\(^2\) Finally, CD95 stimulation of NPDA cells expressing a mutated inactive ASM showed translocation of this protein to the cell surface but no clustering. Fluorescence microscopy studies on those NPDA cells similarly showed a dispersed surface pattern of translocated ASM suggesting that ASM detection by these antibodies is not restricted to aggregated ASM (data not shown).

Cell surface ceramide was immunodetected using the anti-ceramide antibody 15B4 raised against C\(_{16}\)-ceramide coupled to bovine serum albumin. Several lines of evidence attest to the specific binding of this antibody to ceramide. ELISA revealed that 15B4 binding to C\(_{16}\)-ceramide is dose-dependent.\(^3\) Further, loading intact cells with C\(_{16}\)-ceramide conferred strong antibody binding to the cell surface. In addition, the antibody does not significantly bind to unstimulated cells in FACs or fluorescence microscopy excluding a substantive reaction with cholesterol, sphingomyelin, or other phospholipids (see Fig. 2, E and F). ASM deficiency also abrogated antibody binding upon CD95 activation are detected. Finally, thin layer chromatography immunostaining of lipid extracts from CD95 stimulated JY cells showed that the antibody binds to a single band co-migrating with C\(_{16}\)-ceramide, effectively excluding significant interaction with other lipids. Consistent with this observation, the antibody did not bind C\(_{16}\)-dihydroceramide or sphingomyelin in immune thin layer chromatography.\(^4\)

In summary, our data provide evidence for a previously unreported biological function of ASM-released ceramide, i.e. the generation of receptor clusters, which constitute an important prerequisite for receptor signaling. Thus, the data may shed light on modifications of rafts that trigger receptor clustering. Ceramide-mediated receptor clustering might be operational in many receptor systems explaining the diverse functions of ceramide ranging from induction of apoptosis to invasion of bacterial pathogens. Preliminary results from our group show that ASM and ceramide not only trigger clustering of CD95 and CD40, but may play a similar role in T-cell receptor/CD3, B-cell receptor or CD28 signaling. In addition, some pathogenic bacteria may employ ASM-mediated receptor clustering for invasion into mammalian cells. Thus, our data suggest sphingolipid-mediated clustering as a generic mechanism for transmembrane signal transmission.

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\(^2\) E. Gublins, unpublished observations.  
\(^3\) R. Kolesnich, unpublished observations.  

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