

Potentialiation of Fas-mediated apoptosis by an engineered glycosylphosphatidylinositol-linked Fas

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Received 27.3.01; revised 11.9.01; accepted 20.9.01
Edited by J Tschopp

Abstract

FasL and TRAIL are apoptotic ligands of the TNF-like cytokines family, acting via activation of the transmembrane death domain containing receptors Fas for FasL, and DR4 or DR5 for TRAIL. A glycosylphosphatidylinositol-linked TRAIL receptor called DcR1 behaves as a decoy receptor inhibiting TRAIL-mediated cell death in several cellular systems. We engineered and stably expressed a chimeric GPI-linked Fas receptor (Fas-GPI) in T-lymphocyte cell lines constitutively expressing functional transmembrane Fas. Surprisingly, despite lacking the death domain region of functional Fas, Fas-GPI was able to significantly increase Fas-mediated cell death triggered by membrane bound or soluble FasL, whereas engagement of Fas-GPI alone did not trigger apoptosis. This potentiating effect, but not transmembrane Fas activation, was selectively inhibited by protein kinase C activation with phorbol esters, demonstrating that Fas-GPI activated a specific synergistic signal transduction pathway. Fas-GPI and transmembrane Fas were localized in distinct membrane compartments, since Fas-GPI, but not transmembrane Fas, was found in the glycolipid-rich membrane microdomains. These results suggest that apoptosis induced by members of this ligand/receptors family may be differentially modulated through other and parallel signalling pathways.

Cell Death and Differentiation (2002) 9, 329–339. DOI: 10.1038/sj/cdd/4400960

Keywords: GPI; Fas; apoptosis; microdomains

Abbreviations: TNF: Tumor necrosis factor; GPI: glycosylphosphatidylinositol; mAb: monoclonal antibody; PIPLC: phosphatidylinositol-specific phospholipase C; PMA: phorbol 12-myristate 13-acetate

Introduction

The Fas ligand (FasL) receptor Fas and the TRAIL receptors are apoptosis inducers which belong to the Tumor Necrosis

Factor (TNF) receptor superfamily. Fas-mediated apoptosis is an important regulator of the immune response as Fas activation has been involved in the negative regulation of the activated lymphocytes via the activation-induced cell death (AICD),^{1–3} and in the deletion of antigen-presenting cells following the contact with the activated T-lymphocytes.^{4–6} Inhibition of Fas-mediated cell death would therefore have for consequences to increase the lifetime of activated T-lymphocytes or of antigen-presenting cells, which may lead to an increased efficiency of the immune response.

Among the TRAIL receptors (for review, see⁷) is one called DcR1, LIT, TRAIL-R3 or TRID^{8–12} which is endowed with an ability to bind TRAIL comparable to that of the functional receptors called DR4 and DR5,^{13,14} but is glycosylphosphatidylinositol-linked (GPI-linked) to the membrane and therefore incapable of transducing any death signal. Several reports showed that DcR1 inhibits TRAIL-mediated cell death when overexpressed in various TRAIL-sensitive cell lines,^{8,9,12,15} and that PIPLC treatment of DcR1 expressing cells cleaves the GPI-linked protein off the membrane and increases susceptibility to TRAIL.^{12,16} Based on the findings described for the TRAIL receptor DcR1, we engineered a chimeric GPI-linked Fas receptor, which we called Fas-GPI. Fas-GPI was stably transfected into human T-lymphoblastoid cell lines, which constitutively express functional transmembrane Fas. In these transfectants, we analyzed whether the co-crosslinking GPI-linked Fas together with transmembrane Fas receptors via FasL would interfere in the apoptotic signal normally delivered via Fas.

Results

Obtention and characterization of Jurkat transfectants expressing Fas-GPI

The cDNA sequence encoding the transmembrane and intracellular regions of the FAS antigen was deleted and exchanged with the sequence encoding the 37 carboxy-terminal amino acids of DAF (Decay Accelerating Factor, or CD55), which is responsible for the glycosylphosphatidylinositol (GPI) membrane targeting of DAF.^{17–21} At the junction between the Fas sequence and the GPI-anchoring region, we intercalated the *c-myc* sequence recognized by the 9E10 monoclonal antibody (mAb). The FasmycDAF chimeric construct, herein called Fas-GPI, was transfected in the human T-lymphoblastoid Jurkat cell line and stable transfectants were isolated by limiting dilution. The Jurkat cell line constitutively expressed transmembrane full length Fas, and was sensitive to the apoptosis induced by FasL. Although it was anticipated that the expression of membrane-bound Fas-GPI would increase the total amount of Fas on the cell surface as analyzed by flow cytometry, a staining with the

anti-*c-myc* antibody 9E10 was used to discriminate between the transmembrane and GPI-linked forms. Three representative transfectants expressing various amounts of Fas-GPI are shown in Figure 1. They were labelled with the anti-*c-myc* mAb, and displayed low, intermediate and high levels of Fas-GPI, while the mock-transfected Jurkat cell line was not labelled with this antibody (Figure 1A). The transfectants were also stained with an anti-Fas mAb (clone 5D7, IgG1). They all displayed an increased expression of Fas when compared to the mock-transfected cell line, which paralleled that noted with the anti-*c-myc* staining (Figure 1C). The same staining pattern was also obtained with three other anti-Fas IgG1 mAbs directed against different epitopes on Fas (B-G27, B-G30 and DX2), as well as with the anti-Fas agonistic IgM 7C11 (data not shown), which ruled out a putative variability in epitope accessibility for the mAbs on different transfectants. When the transfectants and the mock-transfected Jurkat were treated with phosphatidylinositol-specific phospholipase C (PIPLC), which specifically cleaves the protein in the GPI anchor, the *c-myc* epitope staining almost completely disappeared (Figure 1B), and the intensity of the Fas staining on the three Fas-GPI transfectants decreased to the basal level obtained with the mock-transfected Jurkat (Figure 1D). These results demonstrated that the Fas-GPI protein was effectively GPI-linked and that the expression of the chimeric Fas did not alter the membrane expression level of the endogenous wild-type transmembrane Fas, which remained unchanged. Therefore, any further change in the sensitivity of the Jurkat Fas-GPI transfectants towards FasL should not be attributable to a change in the expression level of endogenous Fas.

Expression of Fas-GPI enhanced susceptibility to cell death induced by membrane-bound and soluble FasL

We next assessed the functionality of the Fas system in these transfectants, and examined whether the expression of Fas-GPI could alter the sensitivity of the Jurkat cells to the apoptosis induced by FasL (Figure 2). The mock-transfected Jurkat cell line as well as the transfectants expressing low, intermediate and high levels of Fas-GPI were labelled with ⁵¹Cr, and incubated with the 1A12 murine effector cells, which stably expressed functional membrane human FasL, or with its untransfected counterpart WR19L, at various effector/target ratios. As expected, the mock-transfected Jurkat cell line was efficiently killed by the FasL-expressing 1A12 cell line, but not by the WR19L FasL-negative control cell line. The Fas-GPI transfectants were also efficiently killed by the 1A12 cell line but not by the WR19L (Figure 2C and A respectively). We noticed that these transfectants were more sensitive to the 1A12 cell line than the mock-transfected cell line, and that their susceptibility to membrane-bound FasL paralleled the expression level of the Fas-GPI truncated receptor. In this 4-h assay, the maximum cell death doubled in the transfectant expressing the highest amount of Fas-GPI when compared to the mock-transfected cells, and the effector/target ratio required to reach 50% of the cell death plateau was diminished around eightfold.

To confirm the involvement of the Fas-GPI receptor in this increased sensitivity to apoptosis, the target Jurkat cells were pretreated with PIPLC in conditions which completely removed the chimeric Fas-GPI from the cell

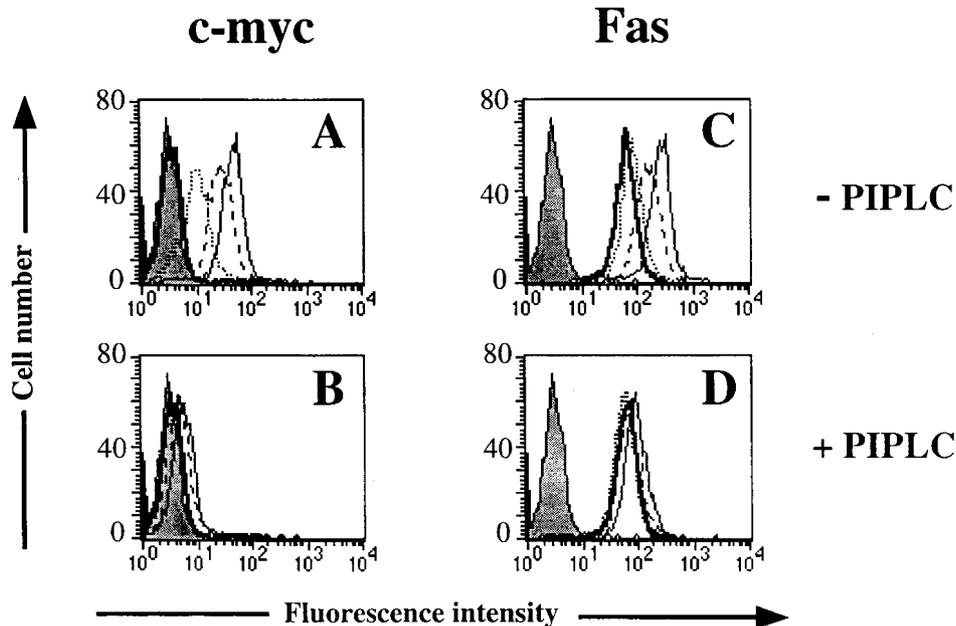


Figure 1 Membrane expression of Fas-GPI, and sensitivity of the GPI anchor to PIPLC. Surface staining for the *c-myc* epitope (A and B) and Fas extracellular domain (C and D) were analysed before (A and C) or after (B and D) PIPLC treatment, for the mock-transfected Jurkat (thick line), and three representative Jurkat transfectants expressing low, intermediate or high amounts of Fas-GPI (dotted, dashed and thin solid line, respectively). The shaded histogram depicts the isotype-matched negative control. This is one representative of four different experiments. These results were reproduced with three other transfectants displaying comparable levels of Fas-GPI

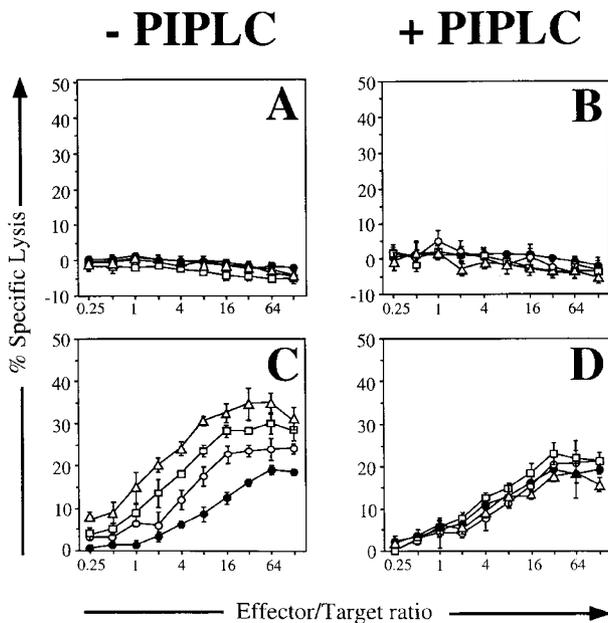


Figure 2 Fas-GPI enhanced Jurkat sensitivity to cytotoxicity mediated by membrane FasL and PIPLC restored basal sensitivity. Mock-transfected Jurkat cell line (filled circles) or the transfectants expressing low (open circles), intermediate (open squares) and high (open triangles) levels of Fas-GPI were treated (B and D) or not (A and C) with PIPLC, labelled with ^{51}Cr and incubated with control WR19L (A and B) or FasL-expressing 1A12 cells (C and D) for 4 h. Cytotoxicity was assessed via measurement of ^{51}Cr release. Results are means of five experiments \pm S.D.

surface (see Figure 1), before the incubation with the effector WR19L or 1A12 cells (Figure 1B,D respectively). Treatment of the mock-transfected Jurkat cell line with PIPLC had no effect on its sensitivity to membrane-bound FasL. In contrast, the sensitivity to FasL of the PIPLC-treated transfectants expressing various amounts of Fas-GPI was drastically reduced, down to the level observed for the mock-transfected Jurkat cell line. Therefore, the gradual increase in sensitivity to FasL of the Fas-GPI transfectants was positively correlated to the increase in their membrane level of Fas-GPI.

We also used purified soluble recombinant FasL (sFasL), to confirm that the dose-dependent apoptosis enhancing effect we ascribed to Fas-GPI was not mediated by another GPI-linked membrane protein constitutively expressed by the Fas-GPI transfectants and mobilized by a specific ligand on the effector cell to enhance the death signal. For this purpose, the Fas-GPI transfectants and the mock-transfected Jurkat cell line were incubated with dilutions of sFasL, and sFasL-mediated cytotoxicity was measured after incubation, with the MTT viability assay carried out on a 20-h period of time. Figure 3 displays the results obtained for two independent transfectants expressing a high level of Fas-GPI. Both Fas-GPI transfectants displayed an increased sensitivity to sFasL, when compared with the control cell line (Figure 3A). When the cells were pretreated with PIPLC before sFasL addition, the sensitivity to sFasL of the mock-transfected Jurkat was not modified, while that of the Fas-GPI transfectants was

decreased close to the level of the mock-transfected cells (Figure 3B). These results confirmed those obtained with membrane-bound FasL and clearly involved Fas-GPI in this enhancing effect while ruling out the putative role of other GPI-linked proteins.

Fas-GPI did not trigger apoptosis on its own

We analyzed the possibility that Fas-GPI could trigger cell death on its own following aggregation via FasL. We took advantage of the *c-myc* epitope to selectively cross-link Fas-GPI with the anti-*c-myc* 9E10 antibody, either directly plastic-coated or aggregated via a polyclonal anti-mouse IgG antiserum. No cytotoxic effect was seen over a wide range of concentrations of the 9E10 antibody (extending from 0.078 ng/ml to 40 $\mu\text{g/ml}$), in the presence or in the absence of various concentrations of the anti-mouse IgG antiserum (from 0.156 ng/ml to 20 $\mu\text{g/ml}$). Conversely, in similar conditions, the isotype-matched B-G27 anti-Fas antibody induced up to $20 \pm 3\%$ ($n=3$) cell death in the transfectant expressing high levels of Fas-GPI (results not shown).

To confirm this result, we stably expressed Fas-GPI or full length Fas into the WR19L cell line, which does not express transmembrane Fas, and transfectants displaying high and comparable expression levels of Fas or Fas-GPI were obtained, as described above for the Jurkat cell line. We verified that Fas-GPI was lipid-linked on the cell surface, since PIPLC induced near complete disappearance of Fas-GPI (Figure 4C) but did not modify the expression of transmembrane Fas (Figure 4B). By using 1A12 effector cells in a cell cytotoxicity assay, we showed that membrane FasL induced significant killing of WR19L cells expressing Fas whereas it had no effect at all on WR19L cells expressing Fas-GPI (Figure 4D). Therefore, Fas-GPI was not able to trigger cell death on its own, demonstrating that it is only capable of potentiating the agonistic effect mediated by the engagement of transmembrane Fas.

Mobilization of Fas-GPI increased Fas-mediated DNA fragmentation and cleavage of caspases 3 and 8

We next analyzed whether Fas-mediated cell death in the presence of Fas-GPI still occurred via apoptosis. Fas-GPI transfected or control Jurkat cells were mixed at a 1:1 ratio with WR19L or 1A12 cells, in the presence or not of the anti-FasL blocking mAb 10F2. After a 2 h incubation, the cell mixture was lysed and DNA fragmentation was analysed by agarose gel electrophoresis. As expected, DNA fragmentation (Figure 5A) occurred for mock-transfected Jurkat cells when incubated with 1A12 cells, whereas it was not detectable in the presence of the control effector cells WR19L. Similar results were obtained for Fas-GPI Jurkat cells, but DNA fragmentation was clearly and reproducibly more pronounced than for mock-transfected Jurkat. For both target cell lines, FasL-mediated death was completely reversed with the FasL specific blocking mAb 10F2, indicating that cell death of the target cell lines was fully mediated by FasL. We next wondered whether the increase

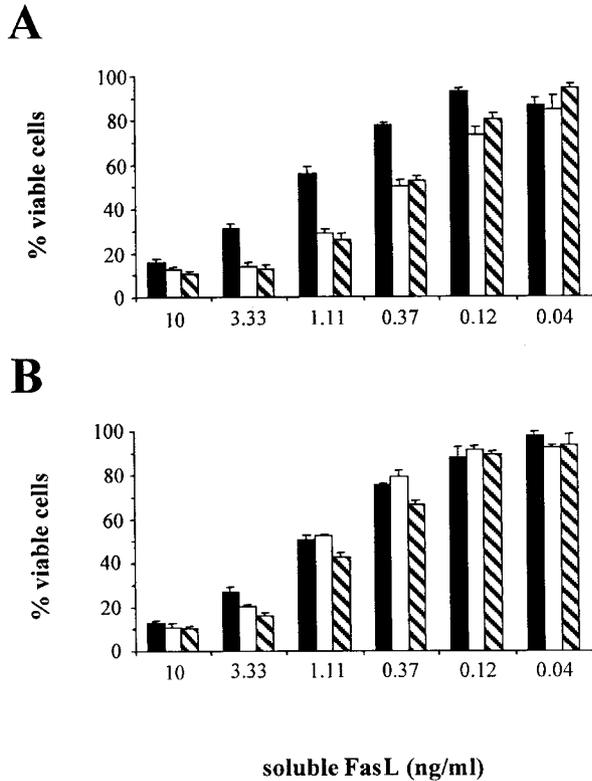


Figure 3 Fas-GPI enhanced Jurkat sensitivity to cytotoxicity mediated by soluble FasL and PIPLC restored basal sensitivity. Mock-transfected Jurkat cell line (filled columns) and two independent transfectants expressing a high level of Fas-GPI (open and hatched columns) were treated (A) or not (B) with PIPLC, before incubation with the indicated concentrations of soluble FasL. Viability was assessed 20 h later with the MTT method, and results were expressed as the percentage of cell viability in comparison to cells incubated in culture medium alone. Results are means of seven experiments \pm S.D.

in DNA fragmentation was associated to an increase in caspase activation. For this purpose, the same experiment was reproduced but aliquots of the cell mixture were lysed at 0, 30, 60, 90 and 120 min following the addition of the effector cells, and the production of the p10 and p14 caspase-8 activation fragments (Figure 5B, upper panel) and p17 and p20 caspase-3 activation fragments (Figure 5B, lower panel) was analyzed by immunoblotting. The activation of caspase-8 and caspase-3 was not detectable for mock-transfected Jurkat cells before 90 min of incubation with 1A12 cells, whereas both caspases were cleaved at detectable levels as early as after 30 min of incubation for the cells expressing Fas-GPI. Therefore, the coengagement of Fas-GPI together with Fas accelerated Fas-mediated apoptosis, leading to a significantly increased DNA fragmentation and cell death.

Fas-GPI and transmembrane Fas were localized in distinct membrane compartments on the cell surface

It is well known that GPI-linked molecules essentially localize in membrane microdomains enriched in cholesterol

and sphingolipids, the so-called lipid rafts, which are characterized by non-ionic detergent insolubility and low density.^{22,23} In contrast, a recent report described that the transmembrane Fas receptor is not localized in these structures.²⁴ Therefore, we hypothesized that Fas and Fas-GPI could reside in distinct subdomains of the cell membrane. To verify this hypothesis, the Jurkat transfectant expressing high level of Fas-GPI was lysed with a non-ionic detergent and the membrane domains were partitioned by sucrose gradient ultracentrifugation. Nine fractions were taken from the top to the bottom of the centrifuge tube. The fractions 2 to 5 presented a visible ring containing the microdomains and the bottom fractions (7 to 9) accounted for the rest of the membranes. Aliquots of each fraction containing identical amounts of total protein were submitted to SDS-PAGE in denaturing conditions and revealed by Western-blotting with specific antibodies as depicted in Figure 6. Consistent with a previous study,²⁵ the transferrin receptor (CD71) was concentrated in the membrane heavy fractions. In contrast, the p56Lck tyrosine kinase was, as previously described,^{23,26-29} mostly localized in the low density fractions containing the microdomains (Figure 6A). The Fas-GPI molecule could be detected using the anti-*c-myc* mAb, and was recovered as a 30 kDa protein exclusively restricted to the microdomains fractions, demonstrating its association with the lipid rafts (Figure 6A). This was confirmed by immunoprecipitation with an anti-Fas mAb followed by western-blotting with a polyclonal anti-Fas antiserum (Figure 6B). In contrast, transmembrane Fas was found as a 45 kDa protein which was exclusively restricted to the heavy membrane fractions (Figure 6B), as it has been recently reported elsewhere.²⁴

The enhancement of cell death mediated by Fas-GPI was inhibited by the protein kinase C activator PMA but not by the src-kinase inhibitor PP1

These findings raised the question of the identification of the biochemical pathway involved in this synergistic proapoptotic signal. Given the well known association between GPI-linked proteins, microdomains and src tyrosine kinases, exemplified in our system by the colocalization of p56Lck and Fas-GPI (see Figure 6), we wondered whether a src kinase specific inhibitor such as PP1³⁰ could counteract the agonistic effect of Fas-GPI. PP1 at 10 μ M was not able to inhibit membrane FasL-induced cell death of mock-transfected Jurkat cells (Figure 7A), although at 1 μ M, it abrogated the anti-CD3-triggered activation-induced cell death (results not shown) as previously found by other authors.³⁰ Therefore, in contrast to the induction of CD3 signal transduction, the Fas pathway did not seem to be sensitive to the inhibition of the src kinase pathway. Similarly, the killing of the Fas-GPI Jurkat transfectants by the FasL effector cells was not affected by PP1 at 10 μ M (Figure 7B).

Phorbol esters such as PMA have been shown to inhibit apoptosis in human T lymphoblastoid cell lines such as Jurkat, by specifically blocking the mitochondrial step of apoptosis.³¹ This step can be triggered by ceramide,

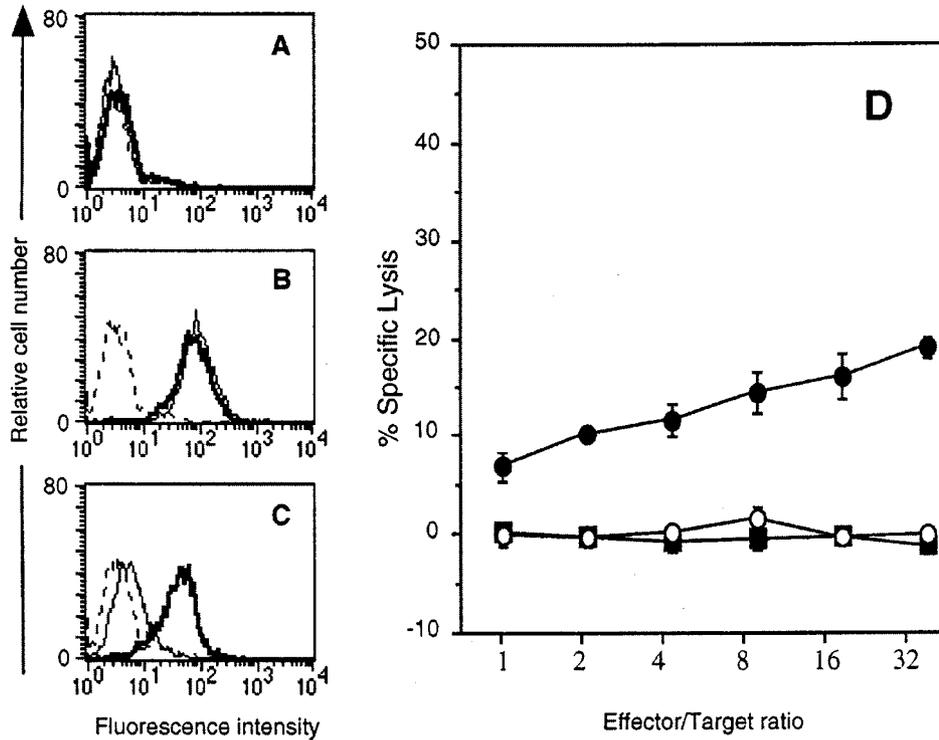


Figure 4 Fas-GPI did not trigger cell death on its own. Fas-GPI or transmembrane Fas was stably expressed in the murine T leukemic WR19L cell line, which is devoid of endogenous Fas. Fas expression was analyzed by flow cytometry on mock-transfected cells (A), one transfectant expressing Fas (B) or Fas-GPI (C), with (thin line) or without (thick line) treatment by PIPLC. Dashed line depicts the isotype-matched negative control. In (D), a ^{51}Cr -release assay was performed in the presence of the 1A12 effector cells, with the mock-transfected WR19L (open circles), one transfectant expressing Fas (filled circles) and one expressing Fas-GPI (filled squares). Three different transfectants expressing almost comparable levels of Fas or Fas-GPI gave similar results

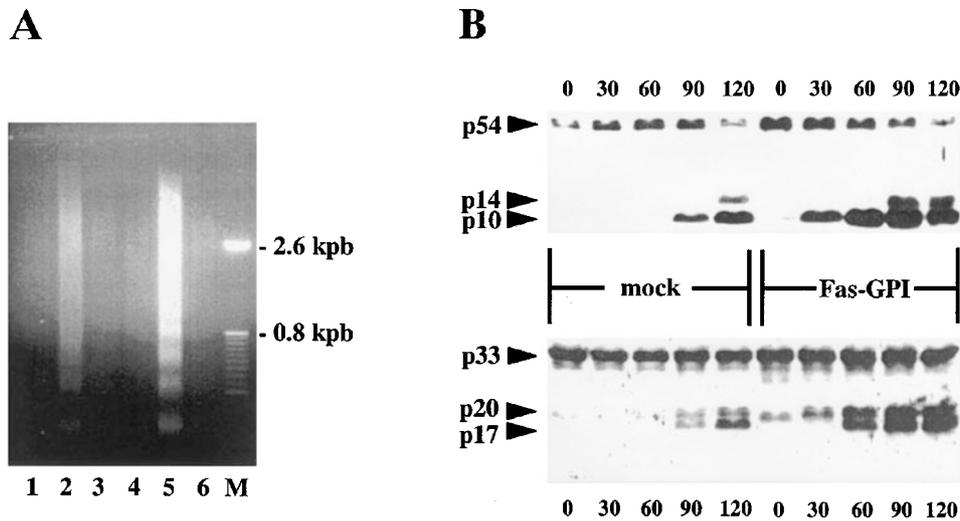


Figure 5 Fas-GPI increase of cell death was mediated by apoptosis. In (A) 3×10^6 mock-transfected or Fas-GPI expressing Jurkat cells were incubated with FasL-expressing (lanes 2, 3, 5 and 6) 1A12 cells or control WR19L cells (lanes 1 and 3) at a 1 : 1 ratio, for 2 h. The incubation was performed in the presence of the anti-FasL neutralizing mAb 10F2 in lanes 3 and 6. Cell mixtures were then harvested and lysed, before DNA fragmentation was analyzed by agarose gel electrophoresis (M is a 50 bp DNA ladder marker). In (B) the cells were lysed at 0, 30, 60, 90 or 120 min after mixing and the presence of caspase 8 (upper panel) and caspase 3 (lower panel) was studied by immunoblot following separation of identical amounts of total proteins ($45 \mu\text{g}$ per lane, corresponding to around 5×10^5 cells). p54 is procaspase 8 and p14 and p10 are its activation products. p33 is procaspase 3 and p20 and p17 are its activation products. A non-specific band migrating between p20 and p17 is visible in the caspase 3 immunoblot

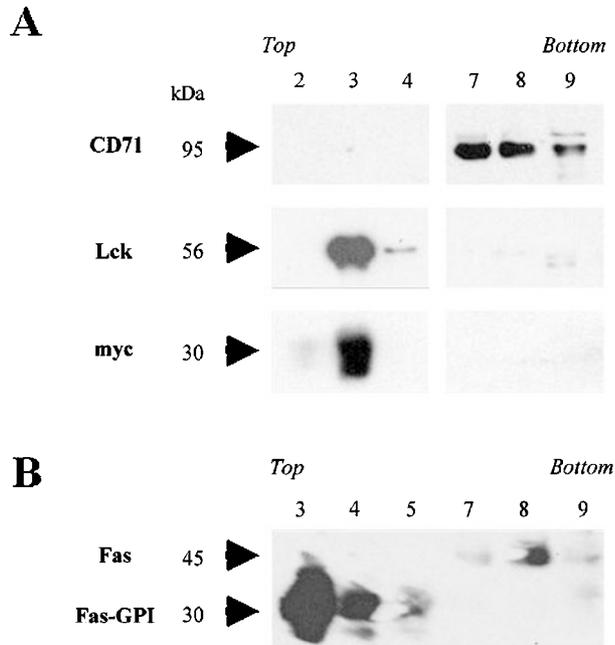


Figure 6 Fas-GPI, but not transmembrane Fas, was associated with the membrane lipid rafts. In **(A)** Jurkat Fas-GPI high was lysed, fractionated on a sucrose gradient as described in Material and Methods, and 10 μ g of total proteins for each indicated fraction were separated by SDS-PAGE before immunoblotting with the indicated antibodies. In **(B)** 50 μ g of total proteins for each indicated fraction from the Jurkat Fas-GPI high were first immunoprecipitated with anti-Fas mAb (5D7), prior to SDS-PAGE and immunoblotting with a goat anti-human Fas polyclonal antiserum

which originates from sphingomyelin, a sphingolipid enriched in microdomains. The mock-transfected Jurkat cell line and the Fas-GPI Jurkat transfectants were preincubated with PMA at 20 ng/ml, and a 51 Cr release assay was carried out using the FasL expressing 1A12 cell line as the effector. In these conditions, PMA had no detectable effect on the FasL-induced cell death of the mock-transfected Jurkat cells (Figure 7C). In contrast, treatment of the Jurkat Fas-GPI cells with PMA induced a substantial decrease in sensitivity to FasL, which returned to levels comparable to that obtained with the mock-transfected Jurkat cell line (Figure 7D). When Fas-GPI was cleaved off with PIPLC before the addition of PMA, the sensitivity of the Jurkat Fas-GPI transfectant to FasL became comparable to that observed for the mock-transfected cells (Figure 7D). Therefore, the Fas-GPI transfectants intrinsically lacked sensitivity to PMA, and Fas-GPI was by itself fully responsible for the acquired sensitivity to PMA. Figure 7 displays the results obtained with the Jurkat transfectant expressing a high level of Fas-GPI, but comparable results were found with the transfectants expressing low and intermediate levels of Fas-GPI (results not shown). Using flow cytometry, we did not detect any variation in Fas or Fas-GPI surface expression on target cells, or any fluctuation in FasL expression on the effector cells, upon treatment with PMA (results not shown). We therefore concluded from these experiments that the Fas-GPI was able to enhance Fas-

mediated cell death, via the activation of a distinct signal transduction pathway specifically inhibitable by PMA, and which did not involve the microdomain associated PP1-sensitive src kinases.

Expression of Fas-GPI also enhanced Fas-mediated apoptosis of the CEM T-lymphoblastoid cell line

To verify that the effect of Fas-GPI was not limited to Jurkat cells, we performed similar experiments with the human T-lymphoblastoid cell line CEM (Figure 8). Cells were stably transfected with Fas-GPI, and transfectants were isolated. In contrast to mock-transfected CEM cells, they displayed surface-bound PIPLC-sensitive Fas-GPI, although at lower amounts than that obtained with Jurkat cells (Figure 8A, for a representative transfectant). As expected, they displayed a higher sensitivity than mock-transfected CEM cells to sFasL mediated cell death (Figure 8B, histogram on the left). Treatment with PIPLC did not alter the sensitivity of mock transfected CEM cells to sFasL, while it partially inhibited cell death of the Fas-GPI expressing cells (Figure 8B, histogram on the right). Therefore, these results confirmed those obtained with the Jurkat cell line.

Discussion

We describe that an engineered GPI-linked form of Fas is capable of enhancing the cell death signal triggered via transmembrane Fas activation by membrane-bound FasL. This effect was confirmed using purified soluble FasL, and was reversed when Fas-GPI was cleaved off the cell surface by PIPLC. Although PIPLC effect appeared weaker towards sFasL than towards membrane-bound FasL, this may simply be due to the requirement for a longer incubation time with the MTT read-out. Indeed, PIPLC-cleaved Fas-GPI will be replaced by newly synthesized Fas-GPI, which might not be shed from the membrane since PIPLC is inactivated by serum components in the culture medium used for the 20-h incubation.

The agonistic effect of Fas-GPI on Fas-induced cell death could be explained by two mechanisms. Firstly, by increasing the total number of Fas binding sites for FasL on the target cell surface, the overall avidity of the FasL/Fas interaction could be augmented, leading to a more efficient delivery of the death signal via transmembrane Fas. Alternatively, Fas-GPI could actively participate to the apoptotic signalling through Fas, via the enhancement of the signal transduction pathway normally triggered by the aggregation of transmembrane Fas, may be through the activation of a distinct but synergistic pathway. This latter possibility was confirmed by the finding that PMA, a protein kinase C activator, was able to selectively abrogate the enhancement of cell death mediated by Fas-GPI without diminishing the response of the Jurkat control cells to the triggering of transmembrane Fas. It is striking that it has previously been reported that mutants of Fas lacking the intracellular death domain behaved as dominant negative receptors.^{32,33} This is consistent with the recent observation that Fas receptors most probably exist on the cell

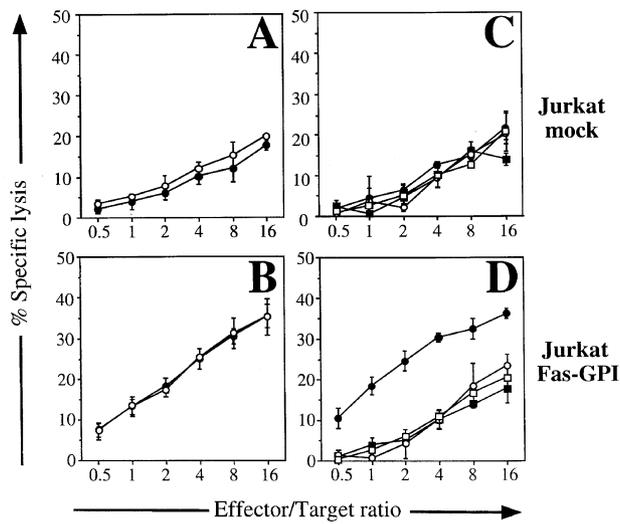


Figure 7 PMA, but not PP1, specifically inhibited the enhancement of cell death mediated by Fas-GPI. Mock-transfected (A and C) or Fas-GPI high expressing (B and D) Jurkat cells were treated (open circles) or not (closed circles) with PP1 (A and B) or PMA (C and D). In addition, in C and D, Jurkat Fas-GPI high were treated with PIPLC before addition of PMA (open squares) or not (closed squares), as described in Materials and Methods

surface as preformed homotrimers,^{34,35} and that the role of FasL is probably to trigger a conformational change of the trimerized Fas receptors to activate the intracellular machinery, instead of reconstituting the functional trimers as previously thought. If truncated Fas molecules lacking the intracellular region enter into the composition of heterotrimers with normal Fas receptors, therefore the triggering step mediated by FasL may be impaired, thereby explaining the dominant negative effect observed. In these cases, the truncated Fas molecules still contained the Fas transmembrane region, whereas Fas-GPI lacks it. Western blot analysis of membranes fractions demonstrated that full length Fas and Fas-GPI were localized in distinct membrane compartments, since no Fas was found in the microdomains fractions which contained Fas-GPI, and reciprocally no Fas-GPI was found in the membrane fractions containing Fas (see Figure 6). This strongly suggests that heterotrimers combining transmembrane and GPI-linked Fas do not exist on the cell surface, in contrast to what happens with death-domain deleted transmembrane Fas. In these conditions, the triggering of Fas might not be impaired, explaining why Fas-GPI did not display an inhibitory effect.

Since Fas-GPI increased Fas-mediated cell death via a PMA-sensitive pathway, we suggest that the glycolipid-rich membrane microdomains could well be involved in the signal triggered by Fas-GPI, as it has been shown in other models, such as T-lymphocyte activation.^{36–38} Microdomains are enriched in tyrosine kinases of the src family, which have previously been involved in Fas-mediated signal transduction by certain authors,³⁹ but not by others.^{40,41} We observed that PP1, a potent and specific inhibitor of tyrosine kinases of the src family, had no blocking effect on both Fas signal transduction and

enhancement of cell death mediated by Fas-GPI, which runs against the involvement of these kinases in Fas-GPI signalling.

Although our system involving Fas-GPI is not physiological as it stands, it strongly suggests that regulatory signalling pathways of Fas-mediated apoptosis exist, which remain to be discovered. A recent report by Sieg *et al.*⁴² described that mutant effector cell lines deficient in ICAM-1 displayed a drastically decreased ability to trigger Fas-induced cell death of targets, in contrast to wild-type ICAM-1 positive cells. Interestingly, LFA-1, the ICAM-1 receptor, is known to be concentrated in the microdomains.²⁶ However, this intimate mechanism is not yet known, and may simply rely on a decrease in cell membrane adhesion, thereby impairing an efficient interaction between Fas and its ligand on the effector cell membrane. In another work, microdomains were shown to be involved in cell death triggered by TNF- α .²⁴ Indeed, the type I TNF receptor was found to be localized in the microdomains, and their disruption blocked TNF- α mediated apoptosis but not Fas-mediated apoptosis.

The agonistic effect exerted by Fas-GPI is in striking contradiction with results obtained with the naturally existing structurally related GPI-linked TRAIL receptor DcR1, which behaves in many cellular models as a decoy receptor for TRAIL, when coexpressed together with functional TRAIL receptors. However, numerous reports did not retrieve any correlation between DcR1 expression and resistance to TRAIL.^{43–49} In several of these studies, intracellular factors such as the caspase 8 inhibitor c-FLIP^{43,46} or as yet unknown factors^{48,49} were implicated as responsible for controlling the TRAIL receptor signal transduction pathway and therefore regulating the sensitivity to TRAIL.^{43,46,48,49} As a consequence, although DcR1 behaves as a decoy receptor in many cellular models, its physiological role is not yet completely known.

To our knowledge, an agonistic effect for DcR1 on TRAIL-induced cell death has never been reported. One of the many possible explanations to our findings with Fas-GPI could be that the membrane localization of DcR1 is different from that of Fas-GPI. DcR1 may not be localized in the microdomains, which would be unusual for a GPI-linked protein, or it may be localized in a distinct subtype of microdomains. Indeed, evidence for the coexistence of various types of microdomains, which harbor different proteins on their surface,^{26,50,51} has been obtained from the study of physical criteria such as size,²⁶ relative resistance to disruption by various non-ionic detergents,⁵⁰ or electron microscopy.⁵¹ Therefore, it is most conceivable that distinct subtypes of microdomains might display different functions in signal transduction.

Experiments are underway to (1) identify constitutively expressed membrane proteins localized into the microdomains, which could behave as the chimeric Fas-GPI in our experimental model, and (2) identify the pathway triggered by Fas-GPI. This would clearly demonstrate the physiological relevance of the phenomenon described here in Fas-mediated apoptosis. The identification of such regulatory pathways could also help to understand why certain cell types, for example the human thymocytes, display an apparent

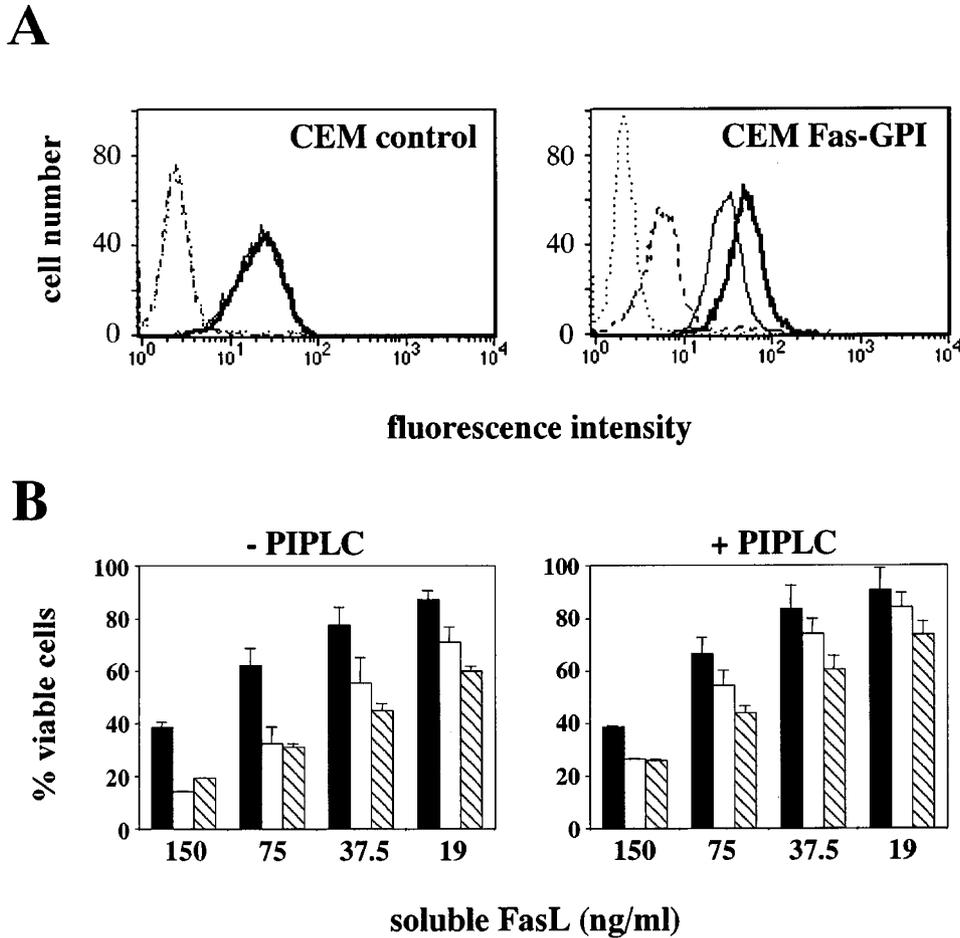


Figure 8 Fas-GPI also enhanced Fas-mediated cell death in CEM cells. In **(A)** surface staining for the *c-myc* epitope (dotted and dashed lines) and Fas extracellular domain (thin and thick continuous lines) were analyzed before (dashed and thick continuous lines) or after (dotted and thin continuous lines) PIPLC treatment, for the mock-transfected CEM (diagram on the left), and one representative CEM transfectant expressing high amount of Fas-GPI (diagram on the right). For the sake of clarity, isotype negative control was omitted, but was superimposed to the dotted lines in both diagrams. In **(B)** mock-transfected CEM (filled columns) and two independent transfectants expressing a high level of Fas-GPI (open and hatched columns) were treated (diagram on the right) or not (diagram on the left) with PIPLC, before incubation with the indicated concentrations of soluble FasL. Viability was assessed 20h later with the MTT method, and results were expressed as the percentage of cell viability in comparison to cells incubated in culture medium alone. Results are means of four experiments \pm S.D.

resistance to Fas engagement despite a strong Fas expression.⁵² Alternatively, the engagement of such costimulation molecules, as well as the use of Fas-GPI, could represent new strategies to modulate Fas-mediated apoptosis.

Materials and Methods

Cell lines and media

The mouse T-lymphoma cell line WR19L and its transfectant 1A12 expressing membrane bound FasL⁵³ were kindly provided by Pr S Nagata (Osaka Bioscience Institute, Osaka, Japan). The human T-lymphoblastoid cell lines CEM and Jurkat 77 were kindly provided by Dr P Anderson (Dana Farber Cancer Institute, Boston, MA, USA). All these cell lines were grown in RPMI 1640 medium supplemented with 8% heat-inactivated fetal calf serum and 2 mM L-glutamine in a 5% CO₂ incubator at +37°C.

Antibodies and other reagents

The fluorescein isothiocyanate (FITC)-labelled goat F(ab')₂ fragment anti-mouse IgG was purchased from Coulter-Immunotech (Marseille, France). Isotype control monoclonal antibody (mAb) 1F10, directed at an irrelevant antigen, was obtained in our laboratory.²⁰ The anti-Fas mAb 5D7 was generated in the laboratory, following immunization of mice with a Fas-Fc fusion protein, and screening of hybridomas' supernatants with a Ba/F3 cell line transfected with the cDNA encoding human Fas. Other anti-Fas antibodies were used, such as B-G27 and B-G30 (Diacclone, Besançon, France), DX2 (Pharmingen, San Diego, CA, USA), and a polyclonal goat anti-human Fas antiserum (R&D Systems Europe, Oxon, UK). The anti-FasL blocking mAb 10F2 was also generated in the laboratory. The anti-*c-myc* mAb 9E10 was purified from supernatants of the hybridoma obtained from Dr Ramsay.⁵⁴ Anti-transferrin mAb was from Zymed Laboratories (San Francisco, CA, USA), anti-p56Lck was from Transduction Laboratories (Lexington, KY, USA), anti-Caspase-3 was from Pharmingen and anti-Caspase-8 was from R&D Systems. Recombinant soluble human FasL (sFasL) and src kinase specific inhibitor 4-amino-5-(4-

methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine (PP1) were from Alexis Corporation (Coger, Paris, France). Phosphatidylinositol-specific phospholipase C (PIPLC) and phorbol 12-myristate 13-acetate (PMA) were from Sigma (L'Isle-d'Abeau-Chesnes, France).

Construction of Fas-GPI and transfection of cells

The plasmid pBS-Fas carrying the full-length human Fas antigen cDNA was a gift from Prof S Nagata. The Chimeric GPI-linked Fas receptor was constructed by subcloning the cDNA sequence encoding the extracellular region of Fas downstream to the *Bam*HI restriction site located 14 nucleotides upstream the start of the transmembrane region, into the pEDr-gp190mycDAF plasmid⁵⁵ digested to remove the gp190 coding sequence. Finally, the Fas-GPI fragment was subcloned into the pBJ1 expression vector¹⁹ which contains the G418 resistance gene. The Jurkat cells, the CEM cells, or the WR19L cells (5×10^6 cells in 0.8 ml) were transfected with 5 μ g of pBJ1-Fas-GPI or empty pBJ.1 vector (=mock-transfected Jurkat cells), or pBJ1-Fas (for WR19L cells only) by electroporation at 280 V for Jurkat and CEM or 290 V for WR19L with a capacitance of 900 μ F using an Easyject+ electroporator (Eurogentec, Seraing, Belgique). Transfected cells were resuspended in 6 ml of growth medium. Twenty-four hours later, G418 (Life Technologies, Cergy-Pontoise, France) was added to the cultures at final concentrations of 1.9, 0.5 and 1.0 mg/ml for Jurkat, CEM and WR19L cells, respectively. After 15 days of G418 selection, the G418-resistant cell population was examined for the expression of Fas antigen and *c-myc* epitope and then cloned by limiting dilution to isolate transfectants expressing the recombinant Fas or Fas-GPI protein.

Flow cytometry analysis of cells

For each staining, 2×10^5 cells were incubated for 30 min at +4°C with saturating concentrations (10 μ g/ml) of the indicated antibody in 0.1 ml of PBS supplemented with 1% Bovine Serum Albumin (BSA) and 0.1% human polyclonal IgG (w/v, both from Sigma). The cells were then washed twice with the same buffer and incubated for 30 min at +4°C with the FITC-conjugated goat anti-mouse IgG. After washing with PBS, the cells were resuspended in 0.14 ml of PBS containing 1% formaldehyde (v/v) and analyzed by flow cytometry with a three color FACScalibur flow cytometer (Becton-Dickinson, Mountain View, CA, USA) equipped with the CellQuest software. For the PIPLC assays, cells (1×10^6) were treated for 1 h at +37°C with one unit of PIPLC in serum-free medium, and were subsequently washed prior to the staining.

Cell cytotoxicity assays

The cytotoxic inducing activity of the membrane FasL-expressing (1A12) or not (WR19L) cells towards the transfected Jurkat or CEM cell lines was determined in a ⁵¹Cr-release assay.⁵⁶ Target cells were labelled with 50 μ Ci of ⁵¹Cr (ICN, Irvine, CA, USA) per 10^6 cells, for 1 h at +37°C. Washed cells were dispensed in round-bottomed 96 well plates (4×10^3 cells per well, in triplicates) and mixed with the effector cells (1A12 cells or WR19L parent cells) at indicated ratios, in a total volume of 0.1 ml. After 4 h at 37°C, 0.025 ml of supernatants were mixed with 0.15 ml of scintillation liquid and the released radioactivity was quantitated using a MicroBeta Trilux beta counter (Wallac, Turku, Finland). The spontaneous release of ⁵¹Cr was determined by incubating the labelled target cells with medium alone, whereas the maximum release was determined by lysing the cells with 1% Triton X-100 in distilled water. The per cent specific lysis was calculated as follows: [(experimental ⁵¹Cr release – spontaneous ⁵¹Cr release)/

(maximum ⁵¹Cr release – spontaneous ⁵¹Cr release)] \times 100. In experiments with PIPLC, the target Jurkat cells were first treated with PIPLC, then labelled with ⁵¹Cr, and washed to remove PIPLC before incubation with effector cells. In experiments with PP1 or PMA, the cells were first labelled with ⁵¹Cr, then incubated for 30 min with the indicated dose of the chemical, and finally mixed with the effector cells. When both PIPLC and PMA were used, the cells were treated for 1 h at +37°C with one unit of PIPLC before the addition of PMA (20 ng/ml).

The cytotoxic activity of human sFasL was measured using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) viability assay. In brief, cells (4×10^4 per well) were cultured for 20 h in flat-bottomed 96 well plates with various concentrations of the apoptosis inducer. Then 0.015 ml of MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h at 37°C. The precipitates were dissolved by adding 0.115 ml of isopropyl alcohol containing 1% formic acid (v/v), and the absorbance was measured at 570 nm (Titertek Labsystems Multiskan reader, Turku, Finland).

For DNA fragmentation assay and for caspase immunoblots, 3×10^6 target Jurkat cells were incubated for 2 h with 3×10^6 WR19L or 1A12 cells. Cells were lysed in 1% Triton X-100 lysis buffer containing protease inhibitors, and the insoluble pellet was discarded after centrifugation. For caspase immunoblots, the supernatant was analysed after protein quantitation. For DNA fragmentation assay, the supernatant was precipitated with 2 volumes of isopropanol, the pellet was washed, dried and resuspended in TE buffer in the presence of RNase, before analysis on a 1.5% agarose gel, as described previously.⁵⁷

Isolation of the membrane microdomains

Membrane microdomains were isolated according to the method of Ko *et al.*²⁴ Briefly, 5×10^8 cells were rapidly chilled in liquid nitrogen and washed with Phosphate-Buffered Saline (PBS). The cells were incubated with 1 ml of HEPES buffer (25 mM HEPES, 150 mM NaCl, pH 7.4) containing 1% Triton X-100 and protease inhibitors (1 mM PMSF, 5 μ g/ml Aprotinin, 10 μ M Leupeptin) for 30 min at 4°C. The lysate was mixed with an equal volume of 85% sucrose (w/v) in HEPES buffer, transferred to a centrifuge tube and overlaid with 5 ml of a 30% sucrose and 3 ml of a 5% sucrose solutions. The sucrose gradient was centrifuged for 21 h +4°C in a Kontron TST4114 rotor at 250000 g_{max} , and nine 1 ml fractions were harvested separately.

Immunoblotting experiments

For membrane fractions and total cell lysates, respectively 10 or 45 μ g of protein were separated by SDS – PAGE in reducing conditions on 12 or 13% gels, and transferred to a PVDF membrane (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was blocked 1 h with TBST (50 mM Tris, 160 mM NaCl, 0.1% Tween 20, pH 8) containing 5% dried skimmed milk, and all subsequent steps were performed in this buffer. The indicated specific antibody was then incubated overnight at 4°C. After four washes, the peroxidase-labelled anti-mouse (Amersham), anti-goat (Vector Laboratories, Burlingame, CA, USA) or anti-rabbit (Zymed) secondary antibody was added for 1 h. Then the proteins were visualized with the enhanced chemiluminescence substrate kit (ECL, Amersham). For the immunoprecipitation experiments, 50 μ g of proteins from the indicated fractions were first immunoprecipitated with 10 μ g of anti-Fas 5D7 mAb in the presence of protein G-Sepharose beads (Sigma) overnight at 4°C. The beads were washed four times in HEPES buffer containing 0.5% Triton X-100 (Sigma). The immunoprecipitated proteins were analyzed by SDS – PAGE as described above.

Acknowledgements

We are indebted to Pr S Nagata who kindly provided us with the WR19L and 1A12 cell lines, and with the cDNA encoding human Fas. We thank Dr P Anderson who kindly provided us with the Jurkat cell line. This work was supported by grants from the Comité des Charentes de la Ligue Nationale contre le Cancer, the Comité de la Gironde de la Ligue Nationale contre le Cancer and the Association pour la Recherche contre le Cancer.

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