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Membrane-Bound Fas Ligand Requires RIP1 for Efficient Activation of Caspase-8 within the Death-Inducing Signaling Complex¹

Michael J. Morgan,^{2*} You-Sun Kim,^{*†} and Zheng-gang Liu^{*}

The serine-threonine kinase RIP1 was originally identified through its ability to bind to the death domain of Fas (CD95). RIP1 has been shown to be recruited to the Fas death-inducing signaling complex (DISC) and is required for the induction of necrotic cell death. In this study, we show that in Jurkat T lymphocytes, RIP1 is also necessary for the most efficient activation of downstream caspases by Fas when treated with membrane-bound Fas ligand, but not with agonistic Abs or cross-linked soluble Fas ligand. RIP1 participates in the Fas-associated death domain protein-mediated recruitment of caspase-8 to the Fas receptor complex in a manner that promotes caspase-8 activation. Cross-linking Abs, such as CH11, bypass the requirement for RIP1 in caspase activation by initiating larger, though less efficient, DISC complexes, while membrane-bound Fas ligand initiates a smaller but more efficient DISC that functions, in part, by effectively incorporating more RIP1 into the complex. Consequently, RIP1 is likely a more integral part of physiological signaling through the Fas/CD95 receptor complex than previously recognized; at least when the signal is mediated by full-length membrane-bound FasL. Cross-linked soluble FasL, which also occurs physiologically, behaves similarly to the CH11 Ab, and may therefore be more likely to initiate nonapoptotic Fas signaling due to less RIP1 in the receptor complex. Thus, agonists that bind the same Fas receptor initiate mechanistically distinct pathways resulting in differential cytotoxicity. *The Journal of Immunology*, 2009, 183: 3278–3284.

The Fas/CD95 receptor is a member of the TNF receptor superfamily and one of the most studied of the death receptors, a subfamily of receptors that contain an intracellular death domain and are capable of mediating cell death and a variety of other signals in their regulatory role of the immune system. The physiological ligand for Fas/CD95 (FasL) is produced by several types of cells, including T cells, as a type II transmembrane protein. Cleavage of the ligand by metallo-proteases in the extracellular portion results in the generation of a soluble portion of FasL (sFasL)³ that lacks the transmembrane domain, but is still capable of trimerization and binding to the receptor, and therefore retains biological activity (1, 2).

Many different agonists have been used to stimulate the Fas/CD95 pathway, and though it is known that all do not cause the same degree of response (3–7), they are often used interchangeably in the literature. Fas was initially discovered as the target of an

IgM mAb (now termed CH11) that had cytolytic activity (8). Therefore, Abs of various kinds have historically been used to ligate Fas and induce apoptosis. Abs to Fas have been shown to be active in vivo, as injection of the Jo2 Ab into mice results in significant liver toxicity due to apoptosis (9). sFasL may be the predominant form of the ligand in vivo that participates in non-apoptotic Fas signaling, and many Fas-expressing cells types do not die in response to sFasL in the absence of a cross-linking agent of some kind. However, the apoptotic potency of sFasL is increased in association with matrix proteins (10). Therefore, when sFasL has been used for cytotoxicity assays, it is usually tagged and cross-linked with an Ab (i.e., Flag-sFasL), or is expressed as a fusion protein that naturally trimerizes (i.e., leucine zipper sFasL). The full-length, membrane-bound Fas ligand (memFasL) is a potent cell death agonist in many cell types and could represent the most physiological ligand for inducing cell death through Fas (2, 11).

Though it has since been mostly studied as part of other pathways, the serine-threonine kinase RIP1 (also known as RIP or RIPK1) was originally identified through its ability to bind to the death domain of Fas (CD95) (12). RIP1 is recruited to the Fas death-inducing signaling complex (DISC) and is required for necrotic cell death initiated by Fas (13). RIP1 is also essential for necrotic cell death initiated by TNF- α , and we have recently shown that that this is due to its participation in the formation of a superoxide-producing complex with the NADPH oxidase NOX1, its adaptor protein NOXO1, TNFR1-associated death domain protein (TRADD) and Rac1 (14). The role of RIP1 in apoptosis has mostly been described as a protective one, since it is important in the activation of NF- κ B and its prosurvival target genes. However, RIP1 has recently been shown to play a critical proapoptotic role in TNF-induced caspase-8 activation in the presence of Smac mimetic, where it nucleates a secondary nonreceptor protein complex containing

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³ Abbreviations used in this paper: sFasL, soluble portion of FasL; memFasL, membrane-bound Fas ligand; DISC, death-inducing signaling complex. FADD, Fas-associated death domain protein; zVAD, N-Benzyloxycarbonyl-Val-Ala-Asp(O-Me) fluoromethyl ketone; siRNA, small interfering RNA; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

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Fas-associated death domain protein (FADD) and caspase-8 (15).

Using Jurkat cells deficient in RIP1, we show in this study that while RIP1 is not absolutely essential for caspase-8 activation and cell death induced by memFasL, it plays a role in the recruitment or organization of caspase-8 in such a way that this caspase is efficiently activated. Cross-linking of the receptor using agonistic Abs or cross-linked soluble FasL bypasses the requirement for RIP1 by producing larger, less efficient complexes that can activate caspase-8 by virtue of the enormous amount of caspase-8 present. Thus, memFasL organizes the Fas DISC in a slightly different way than Ab cross-linking does, resulting in effective RIP1 recruitment and more efficient caspase-8 activation, and leading to a differential sensitivity to cell death by agonists of the same receptor.

Materials and Methods

Reagents

MemFasL and neutralizing anti-Fas (ZB4) were from Upstate Biotechnology. Mouse anti-Fas IgM (CH11), MBL Goat (C20, for IP), and rabbit anti-caspase-8 (H134, Western blot), mouse anti-Fas (B-10), and rabbit anti-Xpress were from Santa Cruz Biotechnology. Mouse anti-RIP1, anti-FADD, and anti-Bcl-2 were from BD Transduction Laboratories. Soluble Flag-FasL, mouse anti-actin, anti-B-tubulin, and anti-Flag Abs, and staurosporine were from Sigma-Aldrich. N-Benzyloxycarbonyl Val-Ala-Asp(O-Me) fluoromethyl ketone (zVAD) was from MP Biomedicals. Rabbit anti-caspase-3, rabbit anti-cleaved caspase-8, mouse anti-Bid, and rabbit anti-FLIP, were from Cell Signaling Technology. Rabbit anti-caspase-8 (cleavage Western blot, supplemental) was from BD Pharmingen. Predesigned Lamin A/C small interfering RNA (siRNA) and RIP1 siRNA pools were obtained from Dharmacon.

Cell culture

Jurkat cells were maintained in RPMI 1640 with 10% FBS, 2 mM glutamine, and 100 U/ml penicillin/streptomycin. Reconstituted cell lines were established by electroporation of RIP1-deficient Jurkat cells with Xpress-RIP1 plasmid and maintained in medium containing 20 mg/ml G418. Clones were isolated using limiting dilution and screened by Western blotting for RIP1 expression and equal expression of DISC components.

Coimmunoprecipitation

Approximately 100 million Jurkat cells were treated with the appropriate agonist. After treatment, cells were lysed in M2 buffer (as in Ref. 14). Lysates were cleared and precipitated with 1 μ g Ab and protein G-agarose beads at 4°C overnight. Beads were washed five times with buffer, boiled in SDS buffer, and then resolved in 4–20% PAGE gels for Western blot analysis.

Cytotoxicity assays

DNA fragmentation was measured using the methodology of Ref. 16. Unless otherwise indicated, all other cell death (or lack of survival) was quantitated using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) reagent (Promega) according to the manufacturer's directions. Statistical analysis was performed using a Student's *t* test (two-tailed).

siRNA knockdown

Oligos were transfected with an Amaxa Biosystems Nucleofector II electroporator with Nucleofector solution V at a concentration of 200 nM, according to the manufacturer's protocol.

Caspase-8 assay

Caspase-8 activity was measured using a colorimetric assay kit (Chemicon) according to the manufacturer's protocol. Activity was normalized to untreated cells of the same type.

Results

RIP1-deficient Jurkat cell are resistant to memFasL

We found that although RIP1-deficient Jurkat cells were resistant to memFasL when compared with the parental Jurkat cell line, they

were equally or slightly more sensitive to a cross-linking IgM Ab (CH11) as seen under the microscope, by propidium iodide exclusion (data not shown), or as measured by DNA fragmentation (Fig. 1A). This was surprising as both agents act on the Fas receptor. To confirm that the cytotoxicity was Fas-mediated, we pretreated cells with a Fas-neutralizing Ab (ZB4), and this effectively inhibited all toxicity due to treatment with either memFasL or CH11, even at high concentrations (data not shown). Although the DNA fragmentation measures the amount of apoptotic cells in a culture at any given time, it often does not account for the many cells that have previously died and fragmented, and are gated out by the flow cytometer as debris. To further confirm the differential sensitivity to the different Fas agonists using a more cumulative measurement of cell death and survival, we did a dose curve comparison of memFasL and CH11 cytotoxicity using the MTS assay. Measurement of death using MTS confirmed what we had found previously, namely that the parental cell line was more sensitive to memFasL (Fig. 1B), while the RIP1-deficient cell line was slightly more sensitive to CH11 treatment (Fig. 1C). Although RIP1-deficient Jurkat cells were derived from the parental Jurkat cell line, we questioned whether there were cell line differences in the downstream caspase-amplification loop. We therefore treated cells with apoptotic stimuli dependent on the intrinsic pathway, namely, staurosporine and UV-C radiation. The dose curve of toxicity to staurosporine was identical (Fig. 1D), suggesting that the mitochondrial pathway was equally proficient in both cell types. RIP1-deficient Jurkat cells were actually slightly more sensitive to UV radiation (Fig. 1E). Therefore, we conclude that the resistance of RIP1-deficient cells to memFasL is not due to a defect in the downstream mitochondrial amplification loop.

We saw no toxicity of sFasL, even at extremely high dose (1 μ g/ml, data not shown), but when cross-linked with an anti-Flag Ab, both cell lines underwent sFasL-induced apoptosis with a similar dose curve (Fig. 1F), suggesting that cross-linked sFasL behaved more like the CH11 Ab and that cross-linking of the receptor by Abs overcomes the potential resistance of the RIP1-deficient cell line to FasL.

Agonist sensitivity correlates with caspase cleavage

As RIP1 has been shown to be essential for necrotic cell death induced by death receptors, including Fas, we first examined whether the Jurkat cell lines underwent cell death in the absence of caspase activity. In the presence of the pancaspase inhibitor zVAD, neither parental nor RIP1-deficient Jurkat cells died in response to memFasL, CH11, or cross-linked sFasL (Fig. S1,⁴ data not shown), suggesting no necrotic cell death was occurring during the treatments.

Cell lines were treated with memFasL or CH11 at three different time points, and cell lysates were probed with an Ab that detects the autocatalytically cleaved forms of caspase-8 that occur in response to stimuli, and which drive the stabilization of enzymatically active caspase dimers (17). In parental Jurkat cells at these concentrations of the two agonists (2 ng/ml FasL, 20 ng/ml CH11), caspase-8 cleavage products were more efficiently generated in response to memFasL than to CH11, as shown by the Western blots (Fig. 2, top, Fig. S2A). However, when the same concentrations were used to treat RIP1-deficient Jurkat cells, cleavage of caspase-8 occurred at comparable levels in both the CH11 and memFasL-treated cells (Fig. 2, bottom, Fig. S2A). Western blotting

⁴ The online version of this article contains supplemental material.

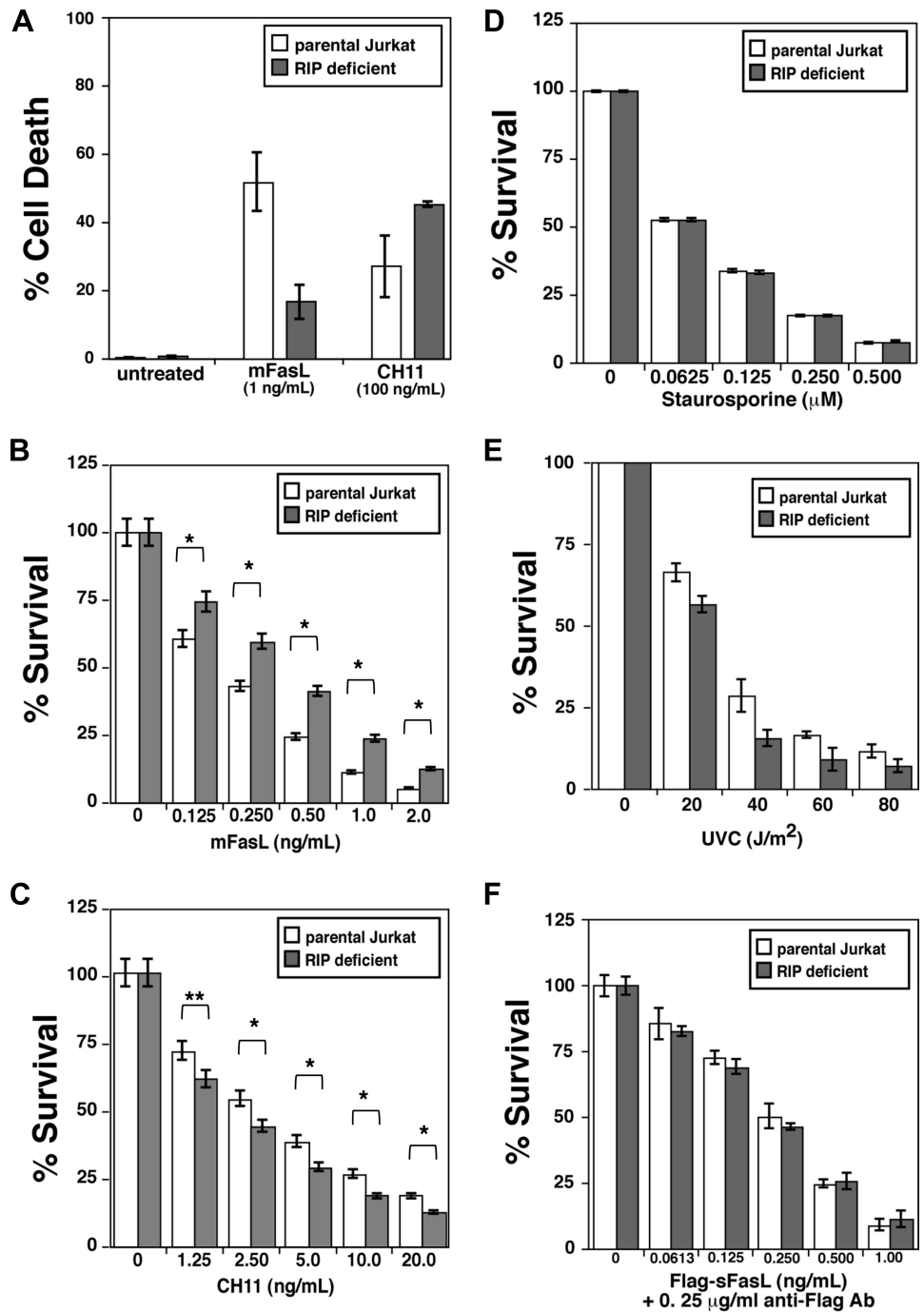


FIGURE 1. RIP1-deficient Jurkat cells are resistant to memFasL, but not to CH11 or other apoptotic stimuli. *A*, DNA fragmentation assay in parental and RIP1 deficient Jurkat cells treated with indicated amounts of memFasL or CH11 Ab for 24 h. *B–F*, Dose curves indicating survival (quantitated using MTS) of parental and RIP1-deficient Jurkat cells treated with memFasL (*B*), CH11 Ab (*C*), staurosporine (*D*), UV-C radiation (*E*), or cross-linked Flag-tagged soluble Fas ligand (*F*) overnight (~18 h). Error bars shown represent SEM, and two-tailed *p* values were calculated using the Student's *t* test. *, *p* < 0.001; **, *p* < 0.01.

of caspase-3 indicated that caspase-3 cleavage occurred preferentially in response to memFasL in the parental Jurkat cells, while CH11 caused slightly more cleavage in the RIP1-deficient cells when compared with memFasL (Fig. 2, Fig. S2B). Thus, while memFasL activates caspases more efficiently than CH11 in the parental Jurkat cells, RIP1-deficient Jurkat cells respond similarly to both agonists at these concentrations, with a slight preference for CH11-mediated caspase activation. The caspase activation indicated by cleavage consequently corresponds to the levels of cell death observed in the two cell types at these doses of agonists.

RIP1 reconstitution restores memFasL sensitivity

To verify the requirement for RIP1 in efficient memFasL-mediated caspase activation and cell death, we made stable cell lines in which RIP1 was reconstituted in the RIP1-deficient Ju-

rat cells. Because high expression of RIP1 was toxic to the cells, we screened a large number of clones to find clones that stably expressed RIP1. Although some clones were isolated that expressed RIP1, none expressed RIP1 to the same extent as the parental Jurkat line (Fig. 3A). Nevertheless, caspase-8 activity was somewhat restored in a majority of them as measured by cleavage of a colorimetric substrate (Fig. 3B), and most RIP1-expressing clones were found to be more sensitive to memFasL than the original RIP1-deficient cell line (Fig. 3C). RIP1-expressing clones that were resistant to memFasL were typically also resistant to CH11 or were significantly deficient in Fas or FADD as detected by Western blot (data not shown). Clones that had no detectable expression of RIP1 were similar to the original RIP1-deficient cells in cytotoxicity assays, or were resistant to both CH11 and memFasL. No clones were isolated

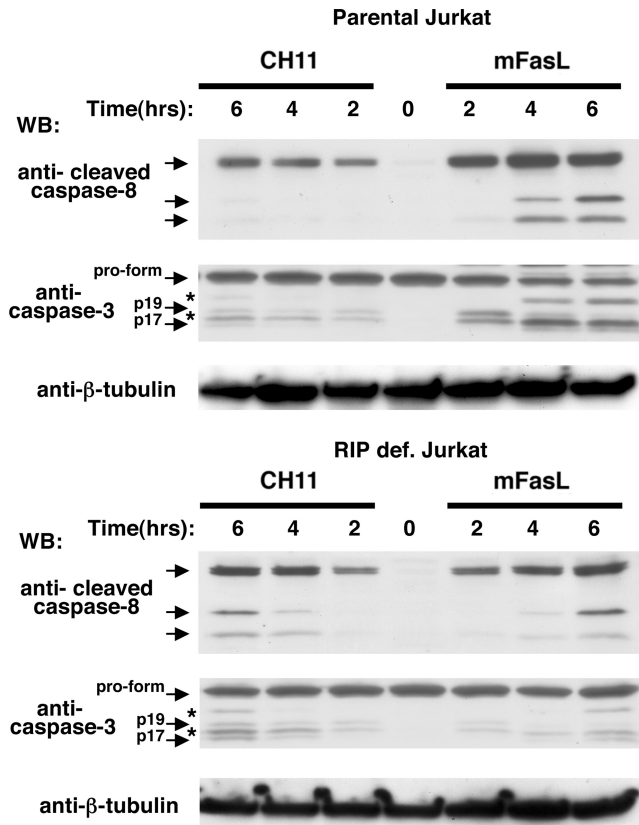


FIGURE 2. Caspase cleavage correlates to cytotoxicity of Fas agonists. Cell lysates from parental and RIP1-deficient Jurkat cells treated with either 2 ng/ml memFasL or 20 ng/ml CH11 for the indicated times and immunoblotted with an Ab to cleaved caspase-8, then stripped and re-probed with caspase-3 and tubulin Abs. Asterisks indicate bands from previous immunoblots. A fresh caspase-3 immunoblot to a different sample is shown in Fig. S2.

that lacked detectable RIP1 expression and that were more sensitive to memFasL than the original RIP1-deficient line. We verified that the RIP1-reconstituted clones used for the cytotoxicity assays had similar levels of many proapoptotic and antiapoptotic proteins (Fig. S3), and while these clones were more sensitive to memFasL than the RIP1-deficient cell line (Fig. 3C), they were not more sensitive to UV and staurosporine (Fig. S4), suggesting that the increased sensitivity upon RIP1 reconstitution was specific to memFasL-mediated apoptosis. Indeed, one clone was actually more resistant to staurosporine when compared with the RIP1-deficient cell line, and yet was more sensitive to memFasL. Thus, we conclude that the reconstitution of RIP1 expression in RIP1-deficient Jurkat cells increases their sensitivity to memFasL and that RIP1 is responsible for the differences seen in response to the different agonists.

To further validate the differential requirement for RIP1 in memFasL- and CH11- mediated cell death, we transfected parental Jurkat cells with siRNA targeting RIP1 or Lamin A/C, and then treated these cells with the two agonists. Although the differences were not as striking as that seen when comparing the RIP1-deficient cell line with the parental cell line, the differences in sensitivity followed a similar pattern as that seen in Fig. 1. Parental Jurkat cells transfected with the RIP1 siRNA were more resistant to memFasL-induced cell death compared with those that had been transfected with the Lamin A/C siRNA (Fig. S5, top). When these same cells were treated with the CH11 Ab, both the RIP1- and Lamin A/C-transfected cells showed nearly identical

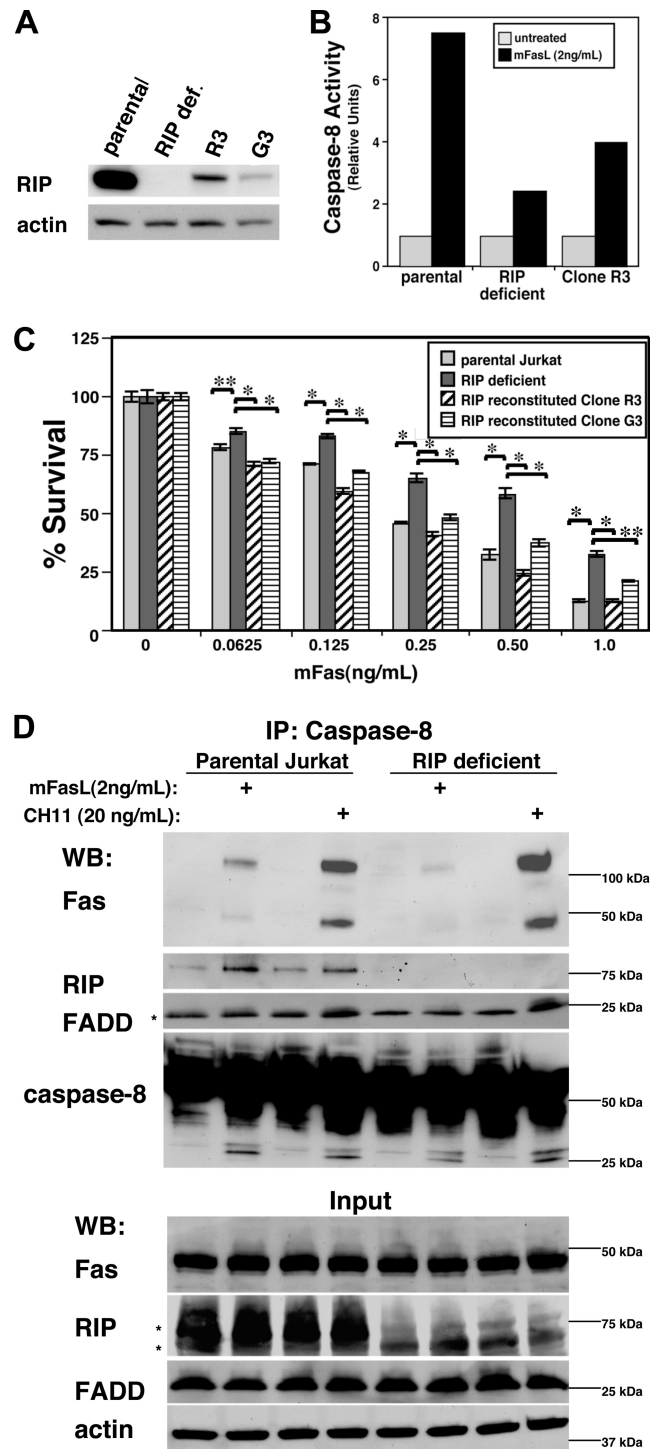


FIGURE 3. RIP1 is partly responsible for recruitment of caspase-8 to the DISC upon memFasL treatment. *A*, Western blots of parental, RIP1-deficient, or RIP1-reconstituted Jurkat cell lines. *B*, Caspase-8 activity assay in parental, RIP1-deficient, or RIP1-reconstituted Jurkat cell lines after 4 h treatment with memFasL. *C*, Cell death assays in parental, RIP1-deficient, or RIP1-reconstituted Jurkat cell lines treated with memFasL overnight (~18 h). Error bars shown represent SEM, and two-tailed *p* values were calculated using the Student's *t* test. *, $p \leq 0.001$; **, $p < 0.01$. *D*, Western blots showing caspase-8 immunoprecipitation (IP) experiments in parental and RIP1-deficient Jurkat cells treated with the indicated amounts of memFasL or CH11 Ab overnight (~18 h).

responses (Fig. S5, bottom), suggesting that RIP1 is not as important in CH11-induced cell death as it is in memFasL-induced cell death.

DISC composition varies with different agonists

As RIP1 activates MAPK in TNF signaling, we used pharmacological inhibitors of JNK, p38, and ERK kinases to see whether they influenced Fas signaling by memFasL and CH11. None of these inhibitors affected the differences in toxicity curves (Figs. S6–S8). In addition, cycloheximide treatment did not prevent the differential cytotoxicity of the agonists, suggesting that changes in gene expression were not involved in the different responses (Fig. S9). The differences did not appear to involve lipid rafts, as treatment with methyl- β -cyclodextrin had no discernable effect (data not shown).

To further investigate mechanistic differences between the agonists, we performed a coimmunoprecipitation of the Fas DISC. Because immunoprecipitation of the receptor itself might not give an accurate comparison due to differences in epitope exposure between the agonists, we immunoprecipitated caspase-8 and probed for the presence of Fas and FADD in the DISC. We pretreated with zVAD to stabilize the complex and prevent caspase-8 release. When treated with the same levels of memFasL or CH11, more Fas was associated with caspase-8 in the memFasL-treated parental Jurkat cell line than in the memFasL-treated RIP1-deficient cell line, while slightly more Fas coprecipitated in the CH11-treated RIP1-deficient cells than in the CH11-treated parental cell line (Fig. 3D). An SDS-stable Fas aggregate was detected in the immunoprecipitates at well above 100 kDa, and followed the same pattern as monomeric Fas. FADD levels in the DISC followed the same pattern as Fas, but were less clear due to the presence of the IgG L-chain band.

These data are consistent with the differences seen in caspase activation when comparing RIP1-deficient and parental cells with the different agonists. In the parental Jurkat cells, we also observed that RIP1 appeared to be more efficiently recruited to the memFasL-initiated DISC than to the CH11-initiated DISC when compared with the levels of Fas in the DISC. However, we were surprised to find that far more Fas was found in association with caspase-8 under CH11-treated conditions than under memFasL-treated conditions in both cell lines. Interestingly, the levels of death induced by 2 ng/ml memFasL were comparable to the levels of cell death induced by 20 ng/ml CH11 in the RIP1-deficient cells (see Fig. 1, B and C). Therefore, at least in RIP1-deficient cells, these levels of agonists induced comparable amounts of cell death even though the amount of caspase-8 in the CH11-induced DISC was estimated to be 10-fold more than that found in the memFasL DISC. This result suggests that although less caspase-8 is recruited to the memFasL DISC, it is activated more efficiently.

Thus, the previous results suggest that memFasL more efficiently activates caspase-8 than CH11, even in the absence of RIP1, that caspase-8 recruitment in response to memFasL is more efficient in the presence of RIP1, and that Ab cross-linking by CH11 overcomes any requirement for RIP1 in recruitment, likely by mediating a large amount of caspase-8 to the DISC, where it is activated relatively inefficiently on a large scale.

We wanted to further test these ideas by performing the experiments in the parental Jurkat cells under conditions where CH11 triggered similar amounts of cell death as memFasL (Fig. 4A). We performed caspase-8 immunoprecipitations in parental Jurkat cells under several different concentrations. Although the levels of RIP1 recruited to the DISC slightly increased with increasing concentrations of CH11 (40 ng/ml–100 ng/ml), RIP1 continued to be more efficiently recruited to the DISC by memFasL treatment when compared with the levels of Fas in the DISC (Fig. 4B). In these Western blots, we used an Fc-specific

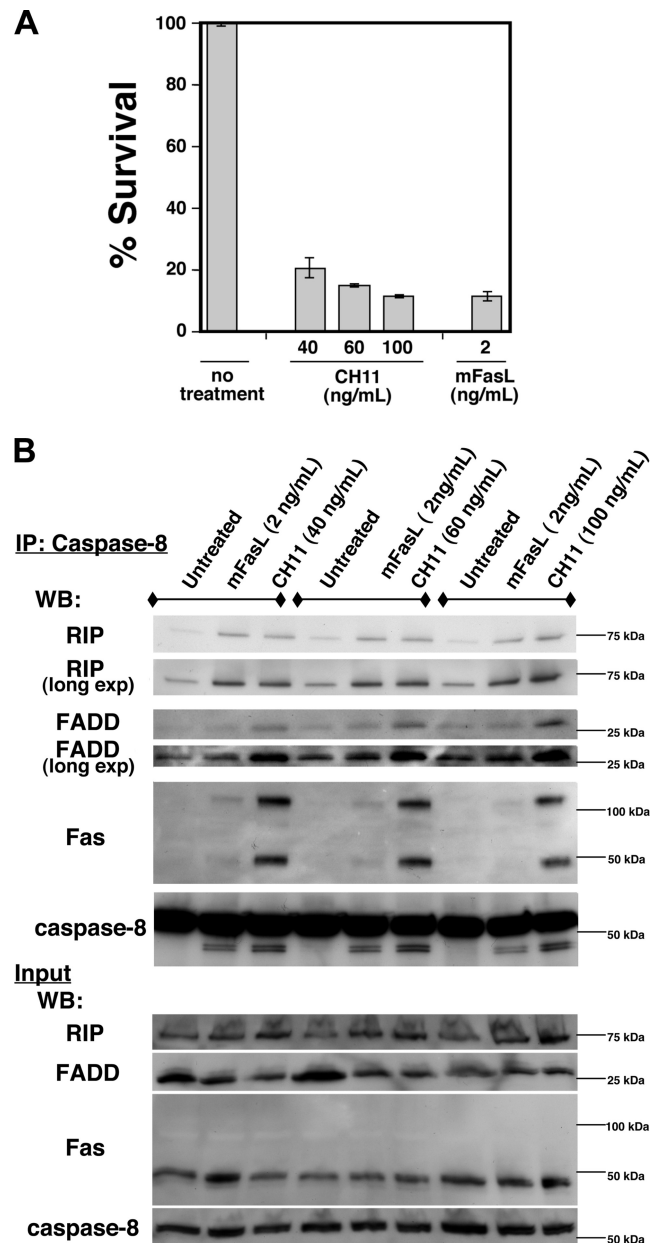


FIGURE 4. At equivalent doses, memFasL is more efficient in RIP1 recruitment than CH11. **A**, Cell death assays of parental Jurkat cells treated with indicated amounts of memFasL or CH11 Ab overnight (~20 h). **B**, Western blots showing caspase-8 immunoprecipitation (IP) experiments in parental Jurkat cells treated with the indicated amounts of memFasL or CH11 Ab overnight (~20 h).

secondary Ab to more effectively visualize FADD. The levels of both FADD and Fas in the DISC with caspase-8 were consistently higher in CH11-treated DISCs than in memFasL-treated DISCs, even though the levels of cell death were similar at 100 ng/ml CH11 or 2 ng/ml memFasL. This supports the hypothesis that memFasL organizes the DISC in such a way that efficient caspase-8 activity is achieved, and also suggests that one of the ways in which efficiency is achieved, in part, is through efficient recruitment and involvement of RIP1. Though CH11 activates the DISC less efficiently, it recruits large amounts of Fas, FADD, and caspase-8, thus increasing the probability of obtaining the proper alignments or conformations of the molecular players within the DISC to trigger caspase-8 activation without RIP1 involvement.

Discussion

Our data suggests that RIP1 is a more integral part of physiological signaling through the Fas/CD95 receptor complex than previously recognized; at least when the signal is mediated by full-length membrane bound FasL. We have shown in this study, among its many roles, RIP1 efficiently enables the FADD-mediated pathway for death induction. Though it is not absolutely essential for cell death, it is, however, likely involved somehow in orienting the molecules of the DISC (Fas, FADD, caspase-8, and others) in such a way that efficient caspase activity is achieved. Thus, the amount of caspase-8 that is recruited to the DISC is not the sole determining factor in how well this caspase is activated, but activation is also dependent on the other DISC components in relation to the amount of caspase-8.

A number of studies in addition to our own clearly indicate that RIP1 is not absolutely essential for either Fas- or TNF-R1-mediated apoptosis (18, 19). Our data show that RIP1-deficient Jurkat cells die, albeit less efficiently, in response to memFasL (Fig. 1B), and other studies have shown that RIP1 knockout MEFs die more rapidly than wild-type MEFs in response to TNF- α (19). Nevertheless, our data is in consistent with recent studies where RIP1 has been proposed to play a significant positive role in caspase-8 activation within the TNF pathway. The remaining amount of TNF-induced death in *Tradd*^{-/-} macrophages is attributed to RIP1 acting as an adaptor molecule to downstream caspase activation (20). In another study, RIP1 is essential for nucleating a secondary complex of FADD and caspase-8 after its release from the TNF receptor (15). Secondary complexes have only recently been postulated to form separately from the receptor in Fas signaling, in conjunction with caspase-8 ubiquitination by CUL3 (21), though CUL3 knockdown has less effect on Fas-induced caspase-8 processing than processing induced by other death receptors. As both RIP1 and caspase-8 are regulated by ubiquitination, it would be tempting to speculate that it is ubiquitin modification that could regulate RIP1–caspase-8 secondary complexes. But although caspase-8 is polyubiquitinated in these secondary complexes (21), only de-ubiquitinated RIP1 is found in secondary complexes associated with caspase-8 (15, 22), and RIP1 is ubiquitinated by cIAPs (22, 23), instead of CUL3. At higher exposure of our Western blots, it appears that RIP1 may be ubiquitinated when coimmunoprecipitated with caspase-8 (Fig. 4B), but we did not probe with an ubiquitin Ab. However, polyubiquitinated caspase-8 secondary complexes are heavily enriched in the lipid raft/cytoskeleton subcellular fraction, and we saw no effect of methyl- β -cyclodextrin on the differences in caspase-8 activation by memFas and CH11, suggesting that raft localization of caspase-8 is not important in the RIP1-dependent differences. Nevertheless, it is possible that memFasL can induce secondary, RIP1-containing, complexes to form, while the CH11 Ab cannot, and this could explain the role of RIP1 in signaling by memFasL. Although we view this as unlikely, we cannot exclude the possibility that we are precipitating two separate complexes, because the ratio of RIP1 to Fas in our memFasL DISC immunoprecipitates would be consistent with this hypothesis.

RIP1 has also been proposed to bind directly to caspase-8 and/or to its noncatalytic homologue, c-Flip (22, 24–27), and has been postulated to even directly activate caspase-8 in the absence of FADD during TNF signaling (27). However, in our hands, FADD-deficient Jurkat cells do not die in response to memFasL (data not shown), and therefore FADD appears to be required for caspase-8 activation, even when it is potentiated by RIP1. This is consistent with our data that suggest that, even in the absence of RIP1, memFasL forms a more efficient DISC with regard to caspase-8 acti-

vation. Nevertheless, it is possible that direct interactions between RIP1 and caspase-8 facilitate a FADD-dependent process, especially within a complex where many protein interactions are likely to occur.

Our data from Fig. 1F suggest that cross-linked soluble FasL acts in a more similar fashion to the CH11 Ab than to the ligand's full-length, membrane-bound form. Thus, it is likely that when sFasL is associated with matrix proteins in a physiological context, it initiates cell death much less efficiently than memFasL due to the recruitment of less RIP1 to the disc. Because of this, it is therefore more likely that sFasL is involved in nondeath signaling pathways, which is consistent with the views of sFasL in the current literature.

That two different Fas agonists act in slightly different ways has implications in modulating death receptor signaling from an investigational therapeutic standpoint. Our data suggests that it is possible to manipulate death receptor pathways using different pharmacological agonists of the same pathway to achieve remarkably different outcomes. The manipulation of Fas signaling is potentially important in developing treatment of autoimmune disease, such as some patients with autoimmune lympho-proliferative syndrome, which have predisposition to lymphoma development as a result of inherited mutations in Fas (28, 29). Other death receptors are also potential targets in the treatment of disease. TRAIL receptor agonists, for example, are currently being tested as anti-cancer agents in clinical trials either individually or in combination with other chemotherapeutics. One would predict, based on our data, that different TRAIL-receptor agonists may have different efficacies, and therefore one may manipulate a given chemotherapeutic window of treatment by selecting a different agonist. Indeed, it has recently been shown that different agonistic Abs to DR5 may act mechanistically distinct from each other in inducing FADD-dependent caspase-8 activation (30). Thus, it seems likely that differential sensitivity of same-receptor agonists could be somewhat common, and may therefore be of benefit in pharmacological modulation of these pathways in the treatment of disease.

In conclusion, we feel that the difference in response to the different agonists in the presence or absence of RIP1 gives some insight into the molecular and structural workings of the Fas receptor complex. As much of the scientific community continues to use Ab and ligand interchangeably despite known differences (3–7), our data is not only a further reminder that the agonists act distinctly and differently, but also at least partially explains why this is the case.

Disclosures

The authors have no financial conflict of interest.

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