

The extracellular domains of FasL and Fas are sufficient for the formation of supramolecular FasL-Fas clusters of high stability

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Using fluorescent variants of Fas and FasL, we show that membrane FasL and Fas form supramolecular clusters that are of flexible shape, but nevertheless stable and persistent. Membrane FasL-induced Fas clusters were formed in caspase-8- or FADD-deficient cells or when a cytoplasmic deletion mutant of Fas was used suggesting that cluster formation is independent of the assembly of the cytoplasmic Fas signaling complex and downstream activated signaling pathways. In contrast, cross-linked soluble FasL failed to aggregate the cytoplasmic deletion mutant of Fas, but still induced aggregation

of signaling competent full-length Fas. Moreover, membrane FasL-induced Fas cluster formation occurred in the presence of the lipid raft destabilizing component methyl- β -cyclodextrin, whereas Fas aggregation by soluble FasL was blocked. Together, these data suggest that the extracellular domains of Fas and FasL alone are sufficient to drive membrane FasL-induced formation of supramolecular Fas-FasL complexes, whereas soluble FasL-induced Fas aggregation is dependent on lipid rafts and mechanisms associated with the intracellular domain of Fas.

Introduction

Fas (Apo-1/CD95) is the prototypic member of the death receptor subgroup of the TNF receptor family. In vivo, Fas is activated by membrane FasL and induces apoptosis in a variety of cells under crucial involvement of its COOH-terminal death domain. The death domain is a protein-protein interaction module found in several apoptosis-related proteins involved in death receptor signaling (Fesik, 2000) and mediates both induction of apoptosis and nonapoptotic signaling pathways (Wajant et al., 2003; Kreuz et al., 2004). At the cell surface Fas forms noncovalently preassembled complexes in which by yet unknown mechanisms the Fas death domains are hindered from interaction with death domain-containing intracellular adaptor proteins (Siegel et al., 2000). Besides membrane FasL, Fas can also be activated by agonistic antibodies or secondarily aggregated soluble FasL. In contrast, native soluble FasL, a pro-

cessed version of membrane FasL, does normally not activate Fas (Suda et al., 1997; Schneider et al., 1998).

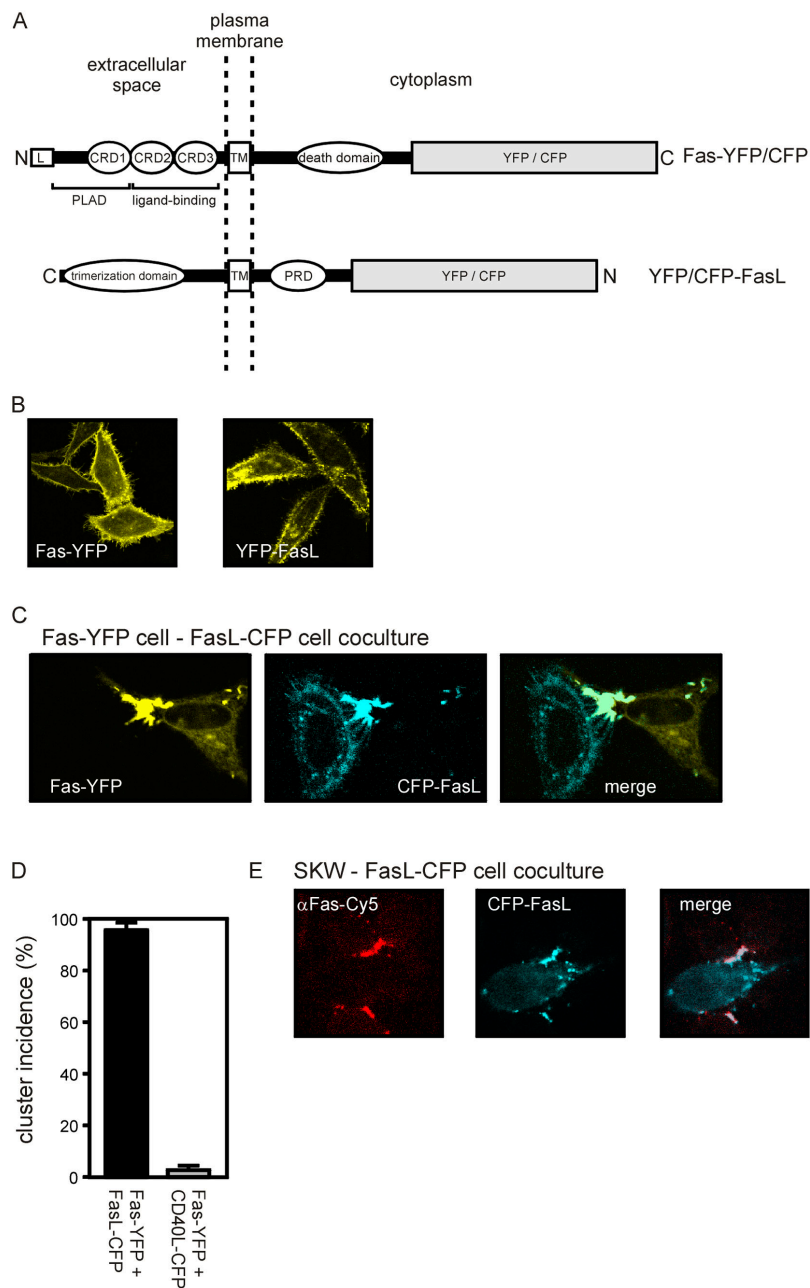
Formation of signaling competent Fas complexes is accompanied by recruitment of the cytoplasmic death domain-containing adaptor protein Fas-associated death domain (FADD; Peter and Krammer, 2003). FADD recruitment depends on the interaction between the death domains of Fas and FADD. Fas-bound FADD in turn is able to bind procaspase-8. Within this death-inducing signaling complex (DISC), procaspase-8 is activated by dimerization (Boatright et al., 2003; Donepudi et al., 2003). DISC-bound active procaspase-8 dimers are then converted by autoproteolytic processing into the mature and active heterotetrameric form of the enzyme which is released from the Fas signaling complex. Active caspase-8 cleaves a limited set of substrates including caspase-3 and the BH3-only protein Bid. Two types of cells can be defined. In type I cells caspase-8 mediated activation of caspase-3 is sufficient to ensure execution of the final steps of apoptosis (Barnhart et al., 2003; Peter and Krammer, 2003). In contrast, in type II cells caspase-8 activation is less efficient and/or activation of effector caspases is inhibited by members of the inhibitor of apoptosis (IAP) protein family (Barnhart et al., 2003; Peter and

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Abbreviations used in this paper: β MCD, methyl- β -cyclodextrin; DISC, death-inducing signaling complex; FADD, Fas-associated death domain; FLIP, fluorescence loss in photobleaching; IAP, inhibitor of apoptosis; ROI, region of interest; SPOTS, signaling protein oligomeric transduction structures.

The online version of this article contains supplemental material.

Figure 1. Membrane FasL and Fas form supramolecular clusters. (A) Scheme of YFP and CFP fusion proteins of Fas and FasL. (B) HeLa cells were transfected with expression plasmids encoding YFP-FasL or CFP-FasL and Fas-YFP or with a mixture of the latter two plasmids in the presence of z-VAD-fmk (20 μ M). Images shown were taken after 24 h and are representative for each experimental group. (C) HeLa cells transfected with CFP-FasL and Fas-YFP, respectively, were harvested 24 h after transfection, mixed at a 1:1 ratio, and cocultured for additional 24 h. Images shown are representative for neighboring cells expressing CFP-FasL and Fas-YFP, respectively. (D) HeLa cells were separately transfected with plasmids encoding the indicated proteins and cocultured overnight. After 24 h, at least 100 yellow fluorescent cells that neighbored one or more blue fluorescent cells were selected on randomly chosen sections of the slide and analyzed for cluster formation. The portions of cells that displayed ligand receptor clusters were determined. (E) HeLa cells were transfected with CFP-FasL and were cocultured 24 h after transfection with SKW cells in the presence of 20 μ M z-VAD-fmk for 3 h. Cells were fixed and stained with anti-Fas and a CY5-labeled secondary antibody.



Krammer, 2003). In these cells, a caspase-8 generated cleavage product of Bid, named truncated Bid, may contribute to apoptosis by inducing Bax/Bak-dependent release of apoptogenic proteins from mitochondria, especially cytochrome *c* and further SMAC/Diablo and HtrA2/Omi (Barnhart et al., 2003; Peter and Krammer, 2003). Cytochrome *c* assembles in the cytoplasm with ATP and the scaffold protein apoptosis promoting factor-1 to form the caspase-9 activating apoptosome (Shi, 2002), which in turn processes and activates caspase-3. Smac/Diablo and HtrA2/Omi block caspase inhibition by members of the IAP protein family (Verhagen and Vaux, 2002). Thus, both mechanisms enhance the effect of initially DISC-activated caspase-8 and facilitate activation of effector caspases, especially caspase-3. A contribution of the mitochondrial pathway to Fas-induced apoptosis has been experimentally defined in vitro by ectopic

overexpression of the anti-apoptotic Bcl2 protein (Scaffidi et al., 1998). In type I cells, Fas-induced apoptosis is not affected by the Bcl2-dependent inhibition of Bax/Bak-mediated release of apoptogenic factors. In contrast, in type II cells Bcl2 expression attenuates apoptosis induction by Fas (Barnhart et al., 2003; Peter and Krammer, 2003). In vivo, thymocytes have consistently been recognized as type I cells. Although some in vivo studies using suboptimal doses of agonistic anti-Fas antibodies found a contribution of the mitochondrial pathway in Fas-induced apoptosis of hepatocytes, the conclusions regarding the in vivo relevance of the mitochondrial pathway for Fas-induced apoptosis extracted from these data are discussed differently (Schmitz et al., 1999; Huang et al., 2000).

Although Fas signaling is well understood, the mechanisms that drive the formation of signaling competent Fas com-

plexes are poorly defined. Soluble trimeric FasL interacts with three molecules of Fas but fails to activate the receptor efficiently. In contrast, hexameric soluble FasL or antibody cross-linked soluble FasL are sufficient to induce Fas signaling (Schneider et al., 1998; Holler et al., 2003). Therefore, it has been suggested that Fas activation requires secondary interaction of trimeric FasL–Fas (FasL₃–Fas₃) complexes. This concept is also in agreement with the observation that agonistic Fas specific antibodies belong to the IgM or IgG3 subclass or require secondary aggregation e.g., by protein A (Kischkel et al., 1995; Huang et al., 1999). Treatment of Fas expressing cells with cross-linked FasL or agonistic anti-Fas antibodies induces higher order clusters (“capping”), which are detectable by immunofluorescent microscopy (von Reyher et al., 1998; Cremesti et al., 2001; Grassme et al., 2001a,b; Algeciras-Schimmich et al., 2002). Although the Fas DISC is rapidly assembled, several lines of evidence suggest that formation of this procaspase-8 converting protein complex is not the first consequence of Fas stimulation but rather a later step in the conversion of preassembled signaling incompetent Fas complexes into “activated” receptor complexes (Grassme et al., 2001a,b; Cremesti et al., 2001; Algeciras-Schimmich et al., 2002). In particular, formation of Fas microclusters (Kischkel et al., 1995; Kamitani et al., 1997; Ruiz-Ruiz et al., 1999; Varadhachary et al., 2001; Algeciras-Schimmich et al., 2002), actin reorganization (Algeciras-Schimmich et al., 2002), inducible or constitutive association with membrane rafts (Grassme et al., 2001a,b; Hueber et al., 2002; Aouad et al., 2004; Muppidi and Siegel, 2004) and acid sphingomyelinase mediated ceramide production (Cremesti et al., 2001) have been discussed as important intermediate steps preceding robust DISC formation.

Here, we show that membrane FasL and Fas form supramolecular clusters of high stability, persisting for several hours. In contrast to early, soluble FasL-induced Fas aggregates, recently named signaling protein oligomeric transduction structures (SPOTS), membrane FasL-induced Fas clusters form independently of the intracellular domain of Fas and occur in the absence of FADD or caspase-8 suggesting that the formation of supramolecular FasL-Fas clusters precedes and is independent of recruitment and activation of downstream signaling components.

Results

Supramolecular FasL-Fas clusters are durable and highly stable

The mechanisms leading to Fas clustering and Fas activation have been predominantly analyzed with cross-linked variants of soluble trimeric FasL or agonistic Fas-specific antibodies. However, the prime *in vivo* activator of Fas is the transmembrane form of FasL. To analyze membrane FasL-induced Fas clustering in living cells by confocal microscopy we have used YFP and CFP fusion proteins of FasL and Fas (Fig. 1 A). Analysis of apoptosis induction, IL8 up-regulation and NFκB activation showed that the various YFP and CFP fusion proteins were functional with respect to apoptotic and nonapoptotic Fas signaling (Fig. S1, available at <http://www.jcb.org/cgi/content/full/jcb.200501048/DC1>). The confocal images of HeLa cells indi-

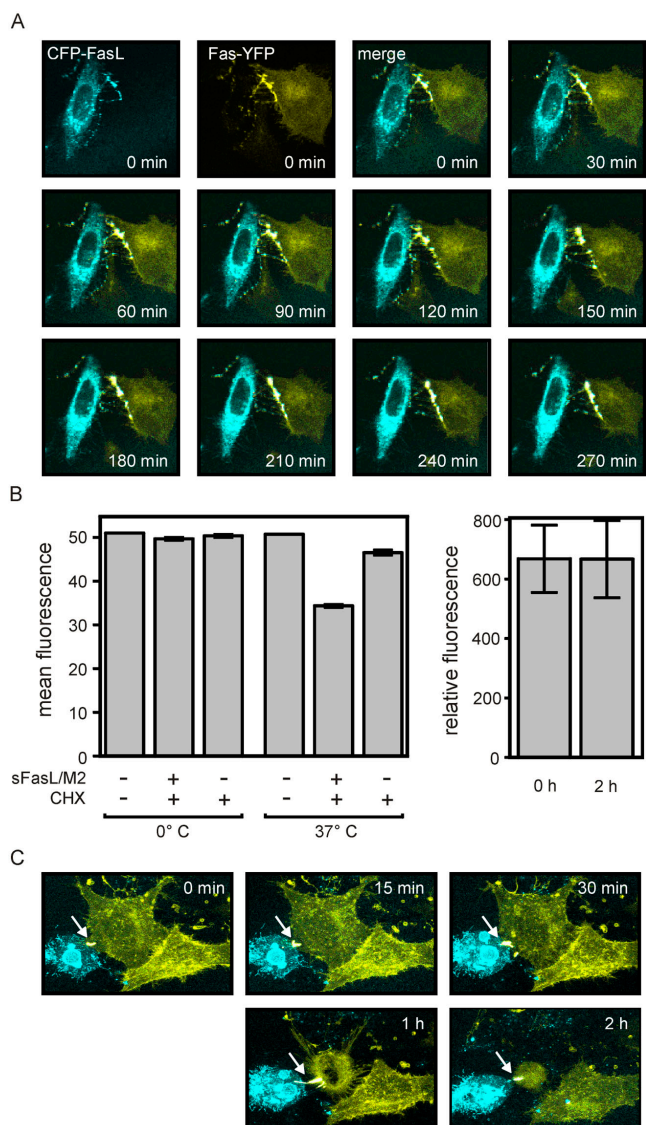


Figure 2. Supramolecular FasL-Fas clusters are highly stable durable structures. HeLa cells were transfected with CFP-FasL and Fas-YFP, respectively, and were cocultured in the presence of 20 μM z-VAD-fmk. The next day, 25 μg/ml CHX were added and after an additional hour a representative pair of transfected cells was analyzed by life imaging fluorescence microscopy. After the indicated times images were taken. (B, left) HeLa cells overexpressing Fas were pretreated with CHX (25 μg/ml) for 30 min and were then challenged with 400 ng/ml of M2-cross-linked Flag-FasL on ice or at 37°C. After 1 h Fas cell surface expression was determined by FACS analysis. The experiment was done in triplicates. (B, right) HeLa cells transfected with Fas-YFP or CFP-FasL were cocultivated. Cells were then pretreated with CHX and fluorescence intensities of nine Fas-YFP expressing cells forming FasL-Fas clusters with neighboring CFP-FasL cells were determined for a 2-h interval by confocal microscopy. (C) HeLa cells were transfected with an expression plasmid encoding Fas-YFP and cultured without z-VAD-fmk. The next day, cells were challenged with a suspension of freshly harvested HEK293 cells, expressing CFP-FasL. Imaging was started when first FasL-Fas clusters became apparent (arrows) and followed until the dying Fas-YFP expressing cell detached from the plastic surface.

vidually transfected with either CFP-FasL or Fas-YFP in Fig. 1 B show the typical membrane expression pattern of these fusion proteins. As expected, both proteins appear as proteins enriched at the plasma membrane and at intracellular membranes and vesicles, en route to the cell membrane. Although Fas-YFP was

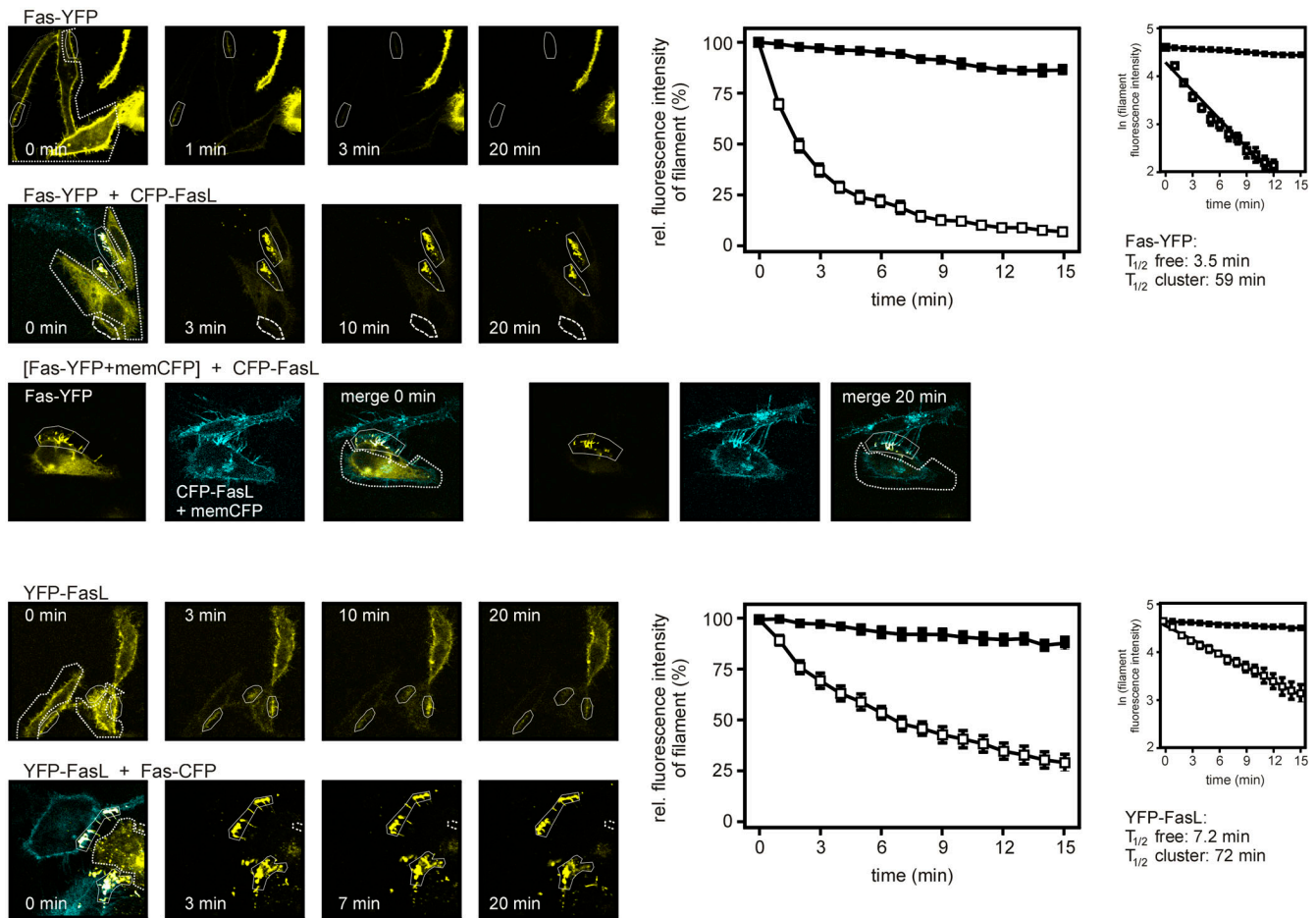


Figure 3. FLIP analysis of free and clustered YFP-FasL and Fas-YFP. HeLa cells transfected with YFP-FasL and Fas-YFP, respectively, were cultured individually or together with HeLa cells expressing CFP fusion proteins of the corresponding receptor or ligand. Images of a representative cell from each experimental group are shown. Bleaching areas covering most of the cell except the analyzed FasL-Fas cluster and/or a control region in the plasma membrane were defined (dotted lines). Cells were initially bleached for 5 min to bleach the majority of the highly diffusible intracellular fraction of the YFP fusion proteins. After this, bleaching was continued, and images were recorded in 1-min intervals. Using these images, loss of fluorescence in nonbleached areas, which contained clusters or control membrane, was determined, as described in Materials and Methods. These areas were defined as ROI, and are indicated (dotted line, bleach area; solid line, ROI of clustered or free YFP fusion protein; dashed line, control ROI). Measurements of clustered YFP fusion proteins (Fas-YFP, closed squares top diagram; YFP-FasL, closed squares bottom diagram) were corrected for each time point for the residual fluorescence of the corresponding "free" protein outside the bleach region. Fluorescence intensities of nonclustered Fas-YFP (open squares top diagram) and YFP-FasL (open squares bottom diagram) determined outside the bleach regions were corrected for the rest fluorescence within the bleach region. Corrected fluorescence intensities were normalized against the relative fluorescence intensity obtained after the initial five bleach cycles. This time point of each experiment was defined as $t = 0$ min. Averaged ($n = 12-15$) normalized relative fluorescence intensities were shown in the diagrams. The fluorescence loss indicating dissociation was linearized by plotting the natural logarithm of the relative fluorescence intensities over time. The third row in the top part shows a control experiment where besides Fas-YFP membrane CFP were coexpressed to demonstrate that the overall morphology of bleached cells was not affected.

found to be homogeneously distributed in the plasma membrane, YFP-FasL (Fig. 1 B) and CFP-FasL (not depicted) showed a speckled distribution. Notably, the detection pattern of FasL did not depend on the individual expression levels of analyzed cells. It is possible that FasL has an intrinsic tendency to form microscopically distinct aggregates. It cannot be formally ruled out that these small aggregates occur partly due to clustering with endogenous Fas. However, the same speckled appearance of YFP-FasL was also observed in cells expressing high amounts of the FasL fusion protein, thus in cells, where the expression level of YFP-FasL is many fold higher than that of endogenous Fas. When CFP-FasL and Fas-YFP were coexpressed in the same cell, both molecules did efficiently colocalize in patches, demonstrating that FasL and Fas can also interact on the same cell (unpublished data). In contrast, patching did not occur when

CFP-FasL was cotransfected with CD40-YFP. In coculture assays of cells separately transfected with CFP-FasL and Fas-YFP (or YFP-FasL and Fas-CFP) FasL-Fas clustering between neighboring cells was found with very high incidence (Fig. 1, C and D). There were no effects on the distribution of CFP-FasL and YFP-Fas when these transfectants were cocultured with CD40-YFP and CFP-CD40L transfected cells, respectively (unpublished data). FasL-Fas clusters at sites of cell-to-cell contacts were formed when Fas-YFP was in type I (SV80, SKW) or type II cells (HeLa, Jurkat), as well as after expression in adherent (SV80, HeLa) and suspended cells (Jurkat, SKW) suggesting that the capability of membrane FasL to induce Fas clustering is not a cell-type specific property (unpublished data). Furthermore, in SKW cells, endogenously expressed Fas molecules were also efficiently incorporated in clusters when the cells were

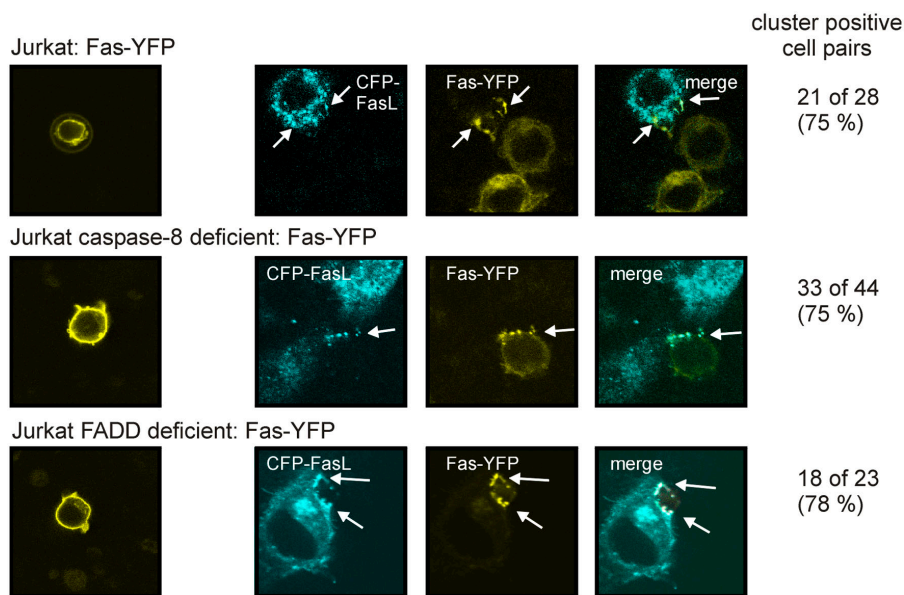


Figure 4. **The cytoplasmic domains of Fas and FasL and caspase-8 and FADD are dispensable for cluster formation.** Parental Jurkat cells and derived clones deficient in FADD or caspase-8 expression were electroporated with Fas-YFP. After 1 d of recovery transfected Jurkat cells were cocultured with CFP-FasL transfected HeLa cells in the presence of 20 μ M z-VAD-fmk. After 3 h Fas-YFP expressing Jurkat cells that overlaid or neighbored CFP-FasL expressing HeLa cells were analyzed for occurrence of FasL-Fas clusters. Total numbers and percentage of cluster positive cell pairs are indicated for one representative experiment. Pictures show examples of isolated Fas-YFP expressing Jurkat cells and Jurkat-HeLa cell pairs having Fas-YFP CFP-FasL clusters (arrows).

cocultured with CFP-FasL expressing cells (Fig. 1 E). Form and size of FasL-Fas clusters between neighboring cells were highly variable. Small “contact plates”, bigger dot-like clusters, and larger membrane stretches of Fas-FasL positive cell-cell contacts were regularly observed. As the majority of the fusion proteins were frequently trapped almost completely within supramolecular FasL-Fas clusters, overall cellular morphology was always ascertained by light microscopy. To prevent induction of apoptosis, FasL-Fas clustering was analyzed in the presence of the pan-caspase inhibitor z-VAD-fmk. This indicates that caspase-8 activation is not necessary for cluster formation.

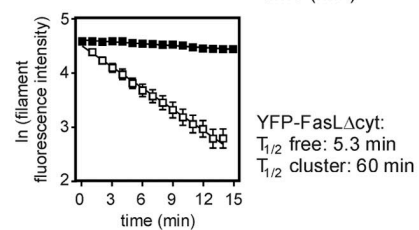
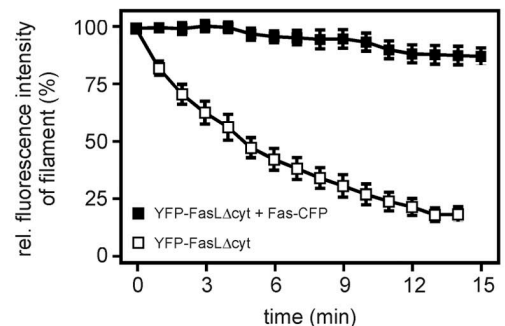
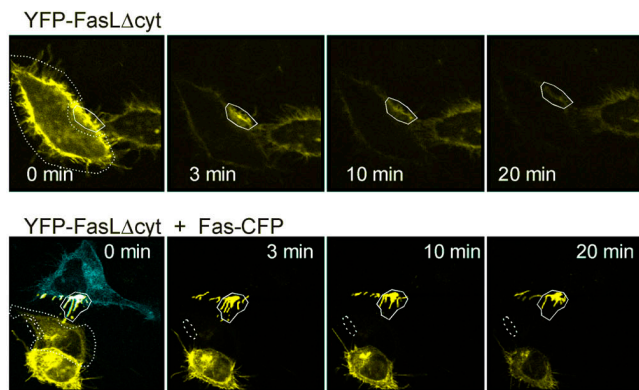
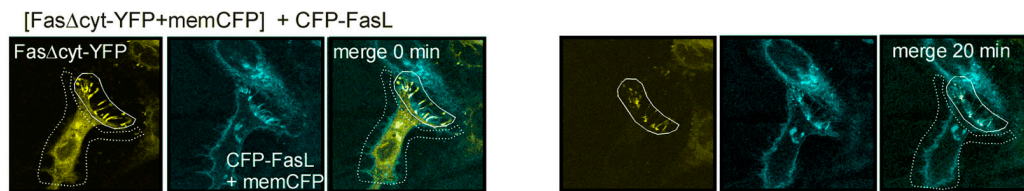
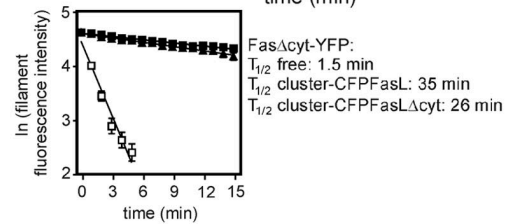
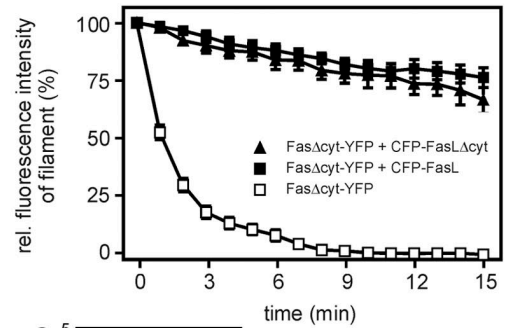
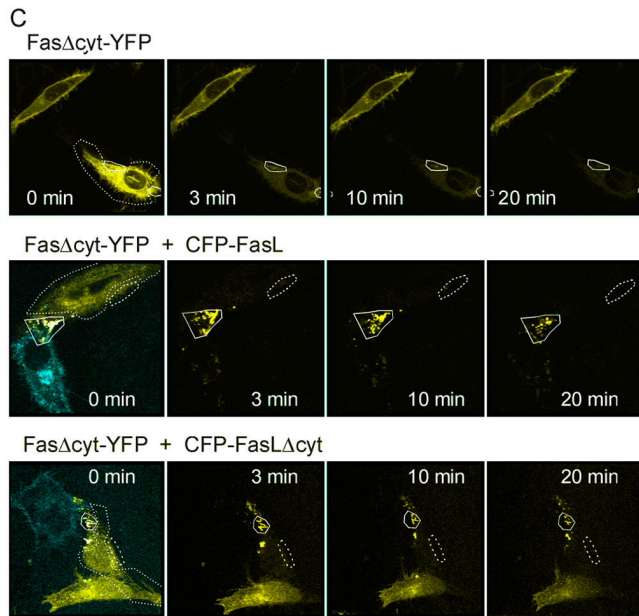
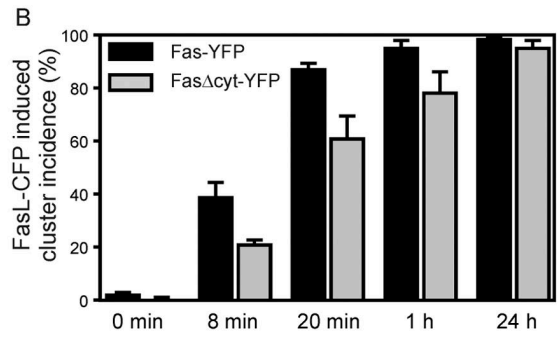
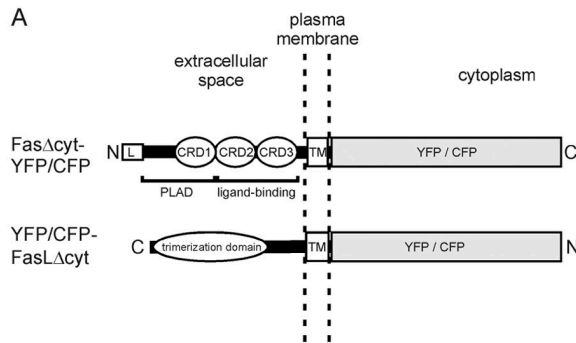
In z-VAD-fmk protected cells time-lapse microscopy revealed that the FasL-Fas clusters, although undergoing slow dynamic morphologic changes, are stable over hours even in the presence of high concentrations of cycloheximide (25 μ g/ml), thus under conditions where synthesis of new proteins was completely inhibited (Fig. 2, A and B). This indicated that under conditions of Fas activation, there was no or only a very limited internalization, an interesting difference to what has been reported for Fas stimulation with soluble reagents (Algeciras-Schimmich et al., 2002; Eramo et al., 2004). To verify whether soluble FasL is capable to induce changes in Fas cell surface expression in the cellular system used here, we comparatively analyzed cell surface expression of soluble FasL-stimulated and nonstimulated Fas transfected HeLa cells. In accordance with soluble FasL-induced internalization, Fas cell surface expression was lowered in the FasL-treated cells by 20–30% 2 h after stimulation (Fig. 2 B). The extent of reduction in Fas cell surface expression was comparable to that observed in the cited studies. To analyze the relationship of cluster formation and apoptosis induction, freshly harvested CFP-FasL expressing cells were added to adherent Fas-YFP transfected cells and cluster formation was monitored online when first signs of cell to cell contact were obvious. Within 1–4 h after initial cluster formation the majority of cycloheximide sensitized (not depicted) as well as a significant portion of nonsensitized (Fig. 2 C) Fas-YFP expressing cells rounded up and detached from the tissue culture plates indicating

apoptosis of Fas-YFP expressing cells. Notably, clusters between CFP-FasL expressing cells and dying Fas-YFP transfectants remained stable and intact till detachment and even longer.

To analyze the stability of the FasL-Fas clusters further, we determined the exchange of unbound and cluster-bound molecules of YFP-FasL and Fas-YFP. We selected transfected cells with moderate YFP fusion protein levels and measured the loss of fluorescence in the FasL-Fas clusters or in plasma membrane areas of comparable size while continuously bleaching the YFP fusion protein in the remaining area of transfected cells (Fig. 3). This revealed a clear difference of fluorescence loss between “free” and clustered fusion proteins. In case of nonclustered YFP-FasL and Fas-YFP, half of the fluorescence was lost in 7.2 and 3.5 min, respectively. Thus, free membrane Fas exerts a significantly higher mobility than nonclustered FasL. However, when YFP-FasL and Fas-YFP were localized in clusters, loss of fluorescence was comparable for both molecules, but strongly reduced, as compared with their nonclustered counterparts. Decay of fluorescence of the clustered proteins was <15% within 15 min. Based on the assumption that the fluorescence of the clustered fusion proteins declines with exponential kinetics the average retention periods were \sim 72 and 59 min.

The extracellular domains of FasL and Fas are sufficient for the formation of supramolecular clusters

The fact that the caspase inhibitor z-VAD-fmk does not interfere with the formation of FasL-Fas clusters in our experiments argues against a crucial role of caspase-8 in FasL-induced Fas aggregation. As studies with soluble Fas agonists have reported such a role we further substantiate this finding by analyzing Fas cluster formation in Jurkat clones deficient in the expression of FADD or caspase-8 after cocultivation with CFP-FasL expressing HeLa cells. Jurkat cells were only transfected with low efficiency (0.5 – 3%). Accordingly, there was only a low number (20–50) of neighboring cell pairs that expressed Fas-YFP and CFP-FasL, respectively. However, clusters were observed in the



vast majority of such cell pairs, indicating that this process is independent from FADD or caspase-8 (Fig. 4). To exclude a contribution of other Fas interacting signaling components in cluster formation, we analyzed the capacity of a Fas deletion mutant lacking the entire cytoplasmic domain (Fas Δ cyt) to form clusters (Fig. 5 A). As in case of the Fas-related CD40-CD40L system, a reverse signaling activity of the ligand has been implicated in the formation of supramolecular clusters (Grassme et al., 2002), we also analyzed deletion mutants of FasL lacking the cytoplasmic domain. Although the Fas Δ cyt mutants were unable to activate the signaling pathways that are associated with Fas and act as dominant-negative variants, the FasL deletion mutants were comparably active as the wild-type ligand (Fig. S2, available at <http://www.jcb.org/cgi/content/full/jcb.200501048/DC1>). In HeLa cells, which has been transfected with Fas-YFP or Fas Δ cyt-YFP, cluster induction was initiated by overlaying FasL-CFP expressing 293 cells. After 1 h coculture near to 100% cluster incidence was observed in Fas-YFP as well as in Fas Δ cyt-YFP expressing cells that were in contact with CFP-FasL expressing cells (Fig. 5 B). This indicates that the extracellular domain of Fas is sufficient to assemble receptor–ligand clusters. However, the cytosolic domain of Fas might exert a modulating effect, as at earlier time points cluster formation was significantly delayed (Fig. 5 B). Notably, Fas Δ cyt-YFP showed also an altered dissociation kinetic, as compared with full-length Fas. When it was clustered with CFP-FasL or CFP-FasL Δ cyt, a significant reduction in $t_{1/2}$ of \sim 50% was observed in both cases (compare Fig. 5 C with Fig. 3). These observations open the possibility that the cytosolic DISC or homotypic interaction of the Fas death domain might further stabilize the ligand–receptor interactions, without having an essential role on its initiation. Similar as the corresponding full-length counterparts, both fluorescent FasL-deletion proteins localized to the plasma membrane. In contrast to the speckled membrane distribution of YFP-FasL (Fig. 1 B), FasL Δ cyt showed a rather homogenous distribution comparable with Fas receptor fusion proteins. Despite these slightly different distributions, there were no significant differences in the mobility of free YFP-FasL Δ cyt and YFP-FasL in the membrane of transfected cells. Furthermore, both proteins showed comparable dissociation kinetics when they were incorporated in clusters with Fas-CFP (Fig. 5 C). Together these data show that the cytoplasmic domains of Fas and FasL, as well as the associated signaling pathways are dispensable for the formation of supramolecular FasL-Fas clusters. Furthermore, the cytoplasmic domain of Fas, but not FasL, has a modulating effect in this process. However, it is possible that the somewhat reduced stability of Fas Δ cyt-YFP clusters reflects a clustering promoting

effect of Fas signaling or the Fas death domain, which may become relevant when Fas agonists of restricted activity, as antibodies or cross-linked soluble FasL, were used.

Lipid rafts and the cytoplasmic domain of Fas have a major role in soluble FasL-induced SPOTS formation, but are dispensable for membrane FasL-induced Fas cluster formation

It has been suggested that constitutive or inducible localization of Fas in membrane areas enriched in cholesterol and sphingolipids (lipid rafts) can be necessary for both clustering and activation of Fas by soluble agonists (Grassme et al., 2001a,b; Hueber et al., 2002; Aouad et al., 2004; Muppidi and Siegel, 2004). Accordingly, Fas signaling was abrogated in various studies by pretreatment of cells with the cholesterol depleting compound methyl- β -cyclodextrin (β MCD; Cremesti et al., 2001; Grassme et al., 2001a,b; Hueber et al., 2002; Muppidi and Siegel, 2004). This raised the question of whether the induction of supramolecular Fas clusters by membrane FasL was dependent of lipid rafts. First, we analyzed association of Fas-YFP and YFP-FasL with lipid rafts in HeLa cells using cell fractionation on sucrose gradients. Transiently expressed lipid raft marker proteins, such as Lck-GFP and a palmitylated YFP variant were found as expected in the detergent insoluble fractions obtained in our experiments (Fig. 6 A). In contrast, cytosolic proteins such as JNK and predominantly cytosolic proteins as tubulin which are not (JNK) or only barely (tubulin) associated with lipid rafts were found in the detergent soluble fractions (Fig. 6 A). Notably, Fas-YFP and YFP-FasL were almost exclusively detected in the detergent insoluble fraction in HeLa cells indicating a constitutive association with lipid rafts (Fig. 6 A). This strong association with lipid rafts was also maintained when both proteins were incorporated in supramolecular clusters. Interestingly, lipid raft association of Fas-YFP was not dependent on its cytoplasmic domain, because the corresponding Fas deletion mutant was also mainly detected in the detergent insoluble fractions (Fig. 6 A). The constitutive association of Fas with lipid rafts was further confirmed by fluorescence microscopy. HeLa cells expressing Fas-CFP were labeled with rhodamine-conjugated cholera toxin B to visualize ganglioside GM1. After patching with an anti-cholera toxin B antibody, strong colocalization in punctuate structures of Fas and cholera toxin was observed in the majority of cells (Fig. S3, available at <http://www.jcb.org/cgi/content/full/jcb.200501048/DC1>). The putative requirement of lipid rafts for the formation of FasL-Fas clusters was then analyzed in cells, which were depleted of cho-

Figure 5. **Cytoplasmic deletion mutants of FasL and Fas are able to form stable clusters.** (A) Scheme of YFP and CFP fusion proteins of Fas and FasL variants lacking the cytoplasmic domains. (B) HeLa cells were either transfected with Fas-YFP or Fas Δ cyt-YFP, grown overnight on cover glasses in 6 well plates and overlaid by centrifugation with FasL-CFP expressing HEK293 cells. Cells were then cocultured at 37°C and fixed after the indicated time intervals. 90 yellow fluorescent cells which were in contact with blue fluorescent cells were analyzed for cluster incidence. The portions of Fas-YFP and Fas Δ cyt-YFP expressing cells, which formed clusters are indicated. (C) FLIP analysis of free and clustered YFP fusion proteins of FasL and Fas variants lacking the cytoplasmic domains. HeLa cells were individually transfected with the indicated YFP and CFP fusion proteins and cocultured in the indicated combinations. FLIP experiments were then performed as described in Fig. 3. Again, dotted lines indicate the bleach areas; solid lines, the ROI of clustered or free YFP fusion proteins; and the dashed lines identify the control ROI. A further control with a cell cotransfected with Fas Δ cyt and membrane CFP is shown to demonstrate maintenance of the overall morphology of bleached cells during bleaching (top, third row).

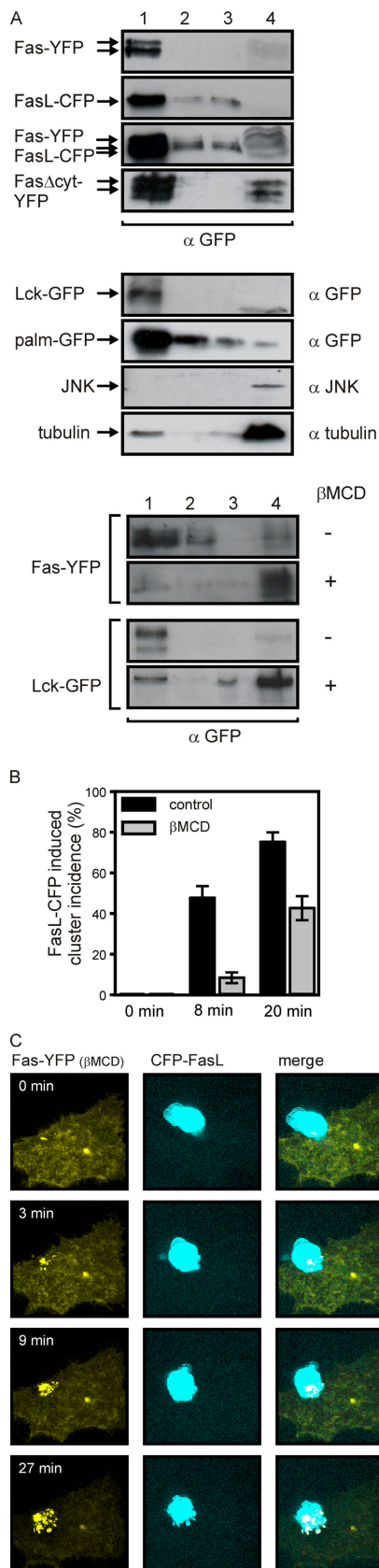


Figure 6. Fas-YFP and CFP-FasL are constitutively associated with lipid rafts. (A) HeLa cells were transiently transfected with the indicated fusion proteins and were grown individually or as cocultures. The next day, cells were analyzed by cell fractionation on sucrose gradients as described in Materials and methods. Four fractions were obtained which are shown here

lesterol by treatment with β MCD. In these experiments, we pre-treated Fas-YFP expressing HeLa cells in serum free medium with 20 mM β MCD for 20 min at 37°C, added freshly harvested CFP-FasL expressing cells and followed cluster formation on-line. Cell fractionation of cholesterol depleted cells on sucrose gradients confirmed that the vast majority of Fas-YFP or Lck-GFP had shifted into the detergent soluble fraction (Fig. 6 A). Successful cholesterol depletion was also evident from the fact that β MCD-treated cells showed significant morphological changes. FasL-Fas clusters were still efficiently formed, when cells expressing the corresponding molecules came into contact, although the assembly of cluster formation was delayed (Fig. 6, B and C). Moreover, clusters were even formed and maintained in cells that were rounded up and detached from the culture dish.

It has been shown in a recent study that cross-linked soluble FasL induce microscopically visible punctuate receptor oligomers in an early stage of Fas-induced apoptosis (Siegel et al., 2004). These structures were termed SPOTS and are possibly related to the supramolecular Fas clusters described in our study. Therefore, we have compared effects of membrane FasL and cross-linked soluble FasL on Fas-YFP aggregation. Although both FasL forms efficiently triggered Fas aggregation (SPOTS or clusters, respectively), some notable differences were also observed. In accordance with the data reported by Siegel et al. (2004), we found that soluble FasL was crucially dependent on the cytoplasmic domain of Fas to induce the formation of SPOTS (Fig. 7, A and B). In contrast, as already shown in Fig. 5, deletion of the cytoplasmic domain of Fas only caused a delay in the kinetics of membrane FasL-induced cluster formation. Furthermore, Fas-YFP SPOTS formation by soluble FasL was completely abrogated in cholesterol depleted cells, whereas membrane FasL-induced cluster formation was only delayed (Fig. 6 B and Fig. 7 A).

Discussion

In previous reports the events of Fas activation have been studied using Fas-specific antibodies, cross-linked soluble FasL (sFasL) or chimeric FasL fusion proteins. However, the physiologic activator of Fas in vivo is membrane FasL. There is further evidence that membrane FasL, soluble FasL, and anti-Fas antibodies do not use exactly the same mechanisms to activate Fas.

from top (1) to bottom (4). Fraction 1, the top fraction corresponded to the lowest sucrose density. This detergent insoluble fraction contained microdomains and lipid rafts. Fraction 4 contained the vast majority of total protein and represented the detergent soluble fraction. The detected proteins and the antibodies used in Western blot analysis are indicated. In experiments shown on the bottom panel, cells had been treated (+) or not (-) with the cholesterol depleting drug β MCD for 20 min before fractionation in order to resolve lipid rafts. (B) HeLa cells expressing Fas-YFP were treated with β MCD for 20 min (gray bars) or left untreated (black bars) and then overlaid with CFP-FasL expressing HEK293 cells by centrifugation. Cells were fixed after the indicated time intervals and the cluster incidence of neighboring cell pairs expressing Fas-YFP and CFP-FasL was determined as described in Materials and methods. The experiment was done in triplicates. (C) HeLa cells were transfected with CFP-FasL and Fas-YFP and grown separately. The next day, Fas-YFP transfectants were depleted for cholesterol (20 mM β MCD, 20 min) in serum-free medium and cocultured with CFP-FasL expressing cells. Online imaging was started when cells came into contact.

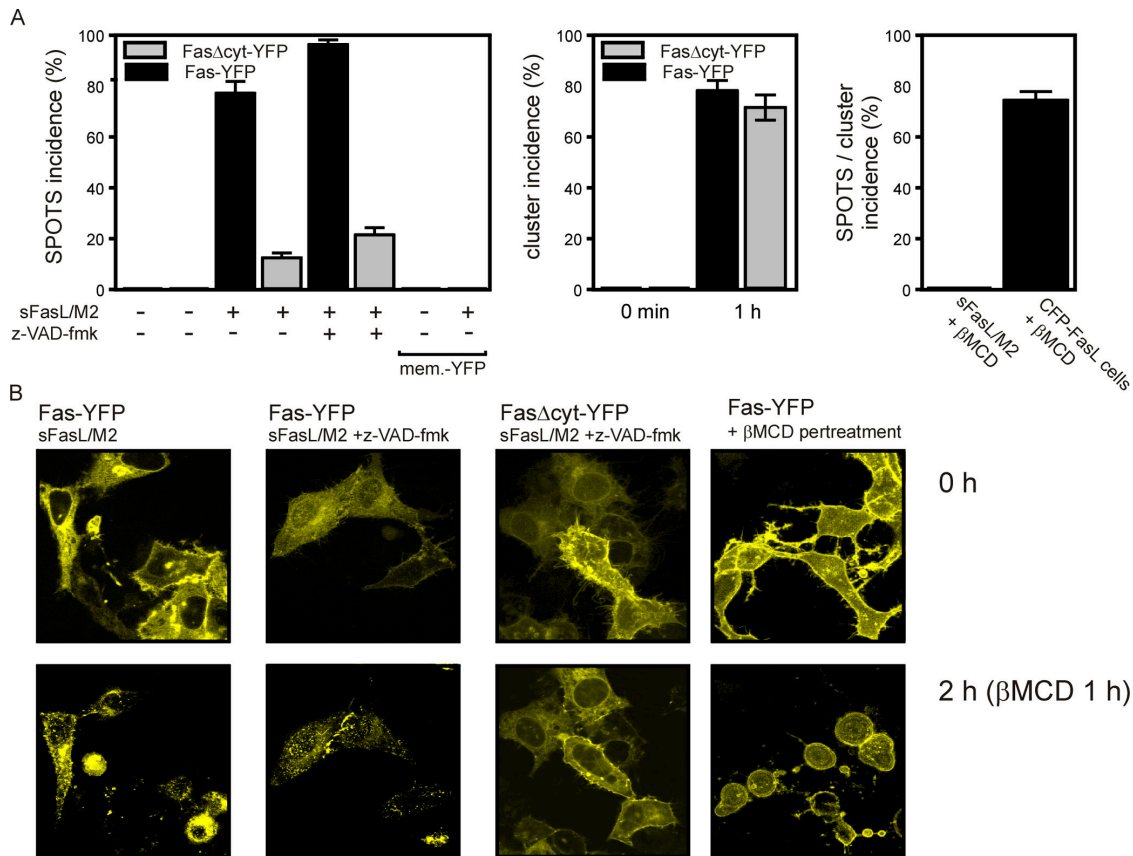


Figure 7. Induction of Fas SPOTS by soluble cross-linked FasL depends on the cytoplasmic domain of Fas and association with lipid rafts. (A, left) HeLa cells were transfected with Fas-YFP, Fas Δ cyt-YFP, or membrane-YFP and stimulated 18 h after transfection with the indicated combinations of cross-linked soluble Flag-FasL (200 ng/ml) and z-VAD-fmk (20 μ M). The proportion of cells that showed SPOTS was determined after 2 h. (Middle) HeLa cells expressing either Fas-YFP (black bars) or Fas Δ cyt-YFP (gray bars) were cocultured with HEK293 cells, transiently transfected with CFP-FasL. After 1 h of coculture, neighbored blue and yellow fluorescent cells were analyzed and cluster incidence was determined as described in Materials and methods. (Right) HeLa cells expressing Fas-YFP were treated for 20 min with 20 mM β MCD, stimulated for with M2-Flag cross-linked soluble Flag-FasL (200 ng/ml), or cocultured with CFP-FasL expressing HEK293 cells. SPOTS incidence and cluster incidence were determined after 1 h. (B) Microscopic images of cells expressing Fas-YFP or Fas Δ cyt-YFP before (0 h) and after stimulation with the indicated reagents (2 h). The image showing Flag-FasL/M2 challenged cells was already taken after 1 h.

For example, earlier studies have found that anti-Fas antibody-induced Fas activation correlates with the formation of SDS-stable Fas microaggregates. It was proposed that this event is a crucial step preceding DISC assembly and Fas signaling (Kischkel et al., 1995; Kamitani et al., 1997; Ruiz-Ruiz et al., 1999; Varadhachary et al., 2001; Algeciras-Schimmich et al., 2002). However, a recent report challenged the main functional relevance of the SDS-stable Fas microaggregates by the observation that these aggregates only occur in antibody-induced Fas activation, but not when Fas activation was achieved by a multimeric FasL fusion protein (Legembre et al., 2003). Moreover, another study found that the serine protease inhibitor TLCK does reduce formation of SDS-stable Fas microaggregates, but nevertheless enhances Fas-induced caspase-8 activation (Lee and Shacter, 2001). It is currently also unclear whether membrane FasL, cross-linked sFasL and anti-Fas antibodies induce Fas clustering in a similar fashion. In particular, some studies reported that Fas clustering induced by cross-linked sFasL or anti-Fas antibodies is a delayed event occurring in an actin- and caspase-dependent, but lipid raft-independent manner (Algeciras-Schimmich et al., 2002), whereas other reports found rapid Fas capping under involvement of lipid rafts (Cremesti et al., 2001; Grassme et al.,

2001a,b). Remarkably, in all these studies, Fas capping/clustering is the consequence of active Fas signaling and initial DISC formation and in so far seems to reflect a positive feedback loop of Fas activated signals on Fas cluster formation.

Using fluorescent fusion proteins of FasL and Fas we describe here that membrane FasL, induces efficient receptor clustering similar to cross-linked sFasL and anti-Fas antibodies. This suggests that the formation of higher order complexes of Fas and FasL is an essential step in membrane FasL-induced Fas activation. Time lapse microscopy and fluorescence loss in photobleaching (FLIP) in living cells revealed that membrane FasL-Fas clusters formed between neighboring cells expressing membrane CFP-FasL and Fas-YFP, respectively, are highly stable and enduring over a period of several hours. Moreover, there was no evidence for internalization of Fas-FasL clusters formed after cell-cell contact (Fig. 2). This is an interesting difference from Fas activation with soluble reagents, where internalization has been observed (Algeciras-Schimmich et al., 2002; Eramo et al., 2004). Therefore, it appears possible that Fas internalization is not a major consequence of Fas activation but rather an effect related to soluble agonists. Moreover, membrane FasL-induced Fas cluster formation at the cell membrane resulted in gene in-

duction or was followed by rapid cell death in the absence of the caspase inhibitor zVAD-fmk (Fig. 7 B; Fig. S1). Thus, our data demonstrate that proper Fas signaling is initiated at the membrane and does not require internalization. The latter may only become relevant when clusters were formed between membrane FasL and Fas, expressed in the same cell or when cluster-bound membrane FasL is shed. The long persistence of membrane FasL-Fas clusters could be of special relevance when FasL expressing cells stimulate apoptosis in resistant Fas expressing cells that can however become sensitive by regulatory mechanisms.

Membrane FasL-induced higher order complexes with Fas were also formed when Fas was expressed in FADD- or caspase-8-deficient cells, in the presence of a broad range caspase inhibitor and when a cytoplasmic Fas deletion mutant was used. Thus, cluster formation occurred under circumstances, where activation of the currently known Fas-induced signaling pathways, in particular caspase-8 activation, was blocked. The concept that Fas induced caspase-8 activation is not an obligate step in robust Fas activation is also in agreement with the signaling capabilities of mutated Fas in *lpr-cg* mice (Desbarats et al., 2003). These mice show normal Fas-induced ERK activation despite a mutation in the death domain of Fas which prevents caspase-8 activation and apoptosis induction. Moreover, an independent Fas deletion mutant has been described, which interferes with FADD binding and apoptosis induction but still has the capacity to trigger JNK activation (Chang et al., 1999).

The relevance of lipid rafts, distinct plasma membrane microdomains enriched in cholesterol and sphingolipids, for Fas-induced DISC formation has been intensively studied. Although some studies found Fas constitutively outside of lipid rafts (Ko et al., 1999; Algeciras-Schimmich et al., 2002, Legler et al., 2003), other reports described significant incorporation of Fas and the DISC components caspase-8 and FADD in such membrane areas constitutively or after stimulation (Grassme et al., 2001a,b; Hueber et al., 2002; Aouad et al., 2004). These discrepancies have been partly attributed to different roles of lipid rafts for Fas signaling in type I and type II cells and may also depend on regulatory events not causally linked to Fas activation. For example, it has been recently shown that TCR signaling in activated CD4⁺ T cells induces relocation of Fas into lipid rafts (Muppidi and Siegel, 2004). Due to the complex and presumably cell type specific role of lipid rafts in Fas activation, we have clarified here the relationship between lipid rafts and the formation of membrane FasL-induced Fas clusters. We found that both Fas-YFP and YFP-FasL were constitutively associated with lipid rafts in HeLa cells (Fig. 6 A). This finding is in agreement with a previous report by Hueber et al. (2002), showing a comparable constitutive raft association of Fas in thymus cells. However, HeLa cells were strongly protected by Bcl2 overexpression against Fas-induced apoptosis (Mandal et al., 1996; unpublished data) and are therefore type II cells, whereas thymocytes are type I cells. Thus, constitutive raft association of Fas does not necessarily correlate with grouping cells into type I and type II. Noteworthy, Fas-YFP remained in association with lipid rafts when it was incorporated into membrane FasL-induced clusters (Fig. 6 A). In our experiments, lipid raft association of Fas did not crucially depend on its cytoplasmic domain suggesting that the Fas

lipid raft association is not related to receptor activation in HeLa cells. The lipid raft association of Fas was not necessary for membrane FasL-induced cluster formation and showed only a modulating effect on this process (Fig. 6 B). In contrast to membrane FasL, cross-linked soluble FasL failed to induce SPOTS after dislocation of Fas from lipid rafts (Fig. 7). Moreover, SPOTS formation was also dependent on an intact cytoplasmic Fas domain (Fig. 7). SPOTS induced by soluble FasL and supramolecular clusters induced by membrane FasL are both activation-associated structures composed of Fas aggregates and are possibly formed by related mechanisms. However, due to the differential relevance of lipid raft association and the cytoplasmic domain of Fas for SPOTS and cluster formation, some differences must exist for the formation of these structures. Based on the experiments with the Fas Δ cyt mutants, it appears that Fas associated signaling events have a crucial role in Fas aggregation during SPOTS formation, but are not essential for Fas aggregation in membrane FasL-induced cluster formation. In fact, as already discussed above, it has been shown that initial Fas-mediated caspase-8 activation is necessary to trigger a positive feedback loop ensuring robust Fas signaling after stimulation with soluble FasL (or agonistic Fas antibodies; Grassme et al., 2001b; Algeciras-Schimmich et al., 2002). This gives rise to the question of why these Fas signaling dependent mechanisms are less important in membrane FasL-induced cluster formation and Fas activation. Perhaps, the spatial orientation and mobility reduction of membrane FasL in the plasma membrane facilitates Fas aggregation, making cytosolic events dispensable for membrane FasL-induced Fas clustering. The absence of Fas signaling may therefore only result in the observed delay in cluster formation. In contrast, it is tempting to speculate that in case of soluble FasL, the signaling dependent mechanisms of Fas aggregation gain higher relevance or are even essential, as cross-linked soluble FasL can possibly not fully substitute for plasma membrane organized FasL molecules. A similar argumentation may also explain the differential relevance of lipid rafts in membrane FasL-induced Fas clustering and soluble FasL-induced SPOTS formation. However, as first Fas resides constitutively in lipid rafts, also in unstimulated cells, and second Fas Δ cyt-YFP is still raft associated, enhancement of Fas aggregation by initial Fas signaling is most likely not related to association of Fas with lipid rafts. Thus, lipid raft association of Fas and a positive feedback of events associated with the Fas cytoplasmic domain may represent two independent mechanisms, compensating for the missing plasma membrane enforced spatial orientation of FasL when its soluble variant is used for Fas activation.

Materials and methods

Reagents, plasmids, and cell lines

cDNAs corresponding to full-length Fas or a deletion mutant containing the extracellular domain and transmembrane domain of Fas were generated by PCR with the expression construct pEF-Bos-Fas as template, using the primers Fas-F, Fas-R and Fas Δ cyt-R with introduce EcoR1 and Sac2 sites (Fas-F: 5'-CCG **GAA TTC** ACA ACC ATG CTG GGC ATC TGG ACC-3'; Fas-R: 5'-TCC **CCG CCG** GAC CAA GCT TTG GAT TTC ATT TCT G-3'; Fas Δ cyt-R: 5'-TCC **CCG CCG** CTT TCT CTT CAC CCA AAC AAT TAG-3').

The amplicons were inserted into the corresponding sites of pEYFP-N1 and pECFP-N1 (CLONTECH Laboratories, Inc.). Similarly, cDNAs encoding full-length FasL or a cytoplasmic deletion mutant of FasL were derived from pcDNA3-FasL by PCR using the primers FasL-F, FasL-R, and FasL Δ cyt-F containing EcoRI and BamHI sites (FasL-F: 5'-GCT TCG AAT TCT ATG CAG CAG CCC TAT AAT TAC CCA-3'; FasL Δ cyt-F: 5'-CCG TCG AAT TCT GGC CTG TGT CTC CTT GTG ATG TTT TTC-3' FasL-R: 5'-CAC GCC GGC GGA TCC GTG CTT CTC TTA GAG CTT ATA TAA GCC G-3'). The amplicons were inserted into the corresponding sites of pEYFP-C1 and pECFP-C1. pEF-Bos-Fas and pcDNA3-FasL were provided by H. Engelmann (Munich University, Munich, Germany). HeLa, HT1080 and HEK293 cells were obtained from ATCC and NCTC cells were a gift from W.S. Wels (Chemotherapeutisches Forschungsinstitut Georg-Speyer-Haus, Frankfurt am Main, Germany). The Jurkat T cell line deficient for FADD and caspase-8 expression and the corresponding parental control cell line were a gift from P. Juo and J. Blenis (Harvard Medical School, Boston, MA) and are described elsewhere (Juo et al., 1998, 1999). The expression plasmid encoding palmitylated CFP was a gift from R.Y. Tsien (Howard Hughes Medical Institute, San Diego, CA). All cells were cultured in DME (GIBCO BRL) supplemented with 5% FCS and grown in 5% CO₂ at 37°C.

Determination of Fas-mediated apoptosis

Cells transfected with the indicated expression plasmids were cultured overnight. The next day, 2.5 μ g/ml CHX was added and after 10 h, cell death was analyzed by crystal violet staining. Alternatively, Fas-YFP expressing cells were stimulated with cross-linked soluble Fas for 2 h. In these experiments, apoptotic effects were analyzed microscopically. Cells that rounded up and/or detached from the cover slide were defined as dying cell. Dying and normal cells were counted to determine the viability of Fas-YFP expressing cells.

Microscopic analysis of living cells or fixed samples

An inverted confocal microscope (model DM IRBE; Leica) with a 63 \times objective (HCX PL APO 63 \times /1.32 oil) was used for online analysis of living cells. The microscope carried a preconditioned chamber (37°C, 5% CO₂) on its stage and was operated by confocal software TCS SL (v. 2.5.1227, serial no. 194043; Leica). Living cells were grown on glass bottom culture dishes, observed over 20 min up to several hours and imaged in regular intervals. Fixed and mounted cells were analyzed using a DM RE confocal microscope with a 100 \times objective (HCX PL APO 100 \times /1.40 oil; Leica). This microscope was operated by confocal software TCS SP2 (v. 2.5.1227; Leica). In all microscopic experiments excitation of CFP fusion proteins was performed at 456 nm, the emission was detected between 470 and 500 nm and visualized blue. Excitation of YFP proteins was done at 514 nm, the emission signal was detected above 600 nm and visualized yellow. Analysis of fluorescent samples, containing both CFP and YFP proteins were done by sequential screening. The absence of nonspecific signals in both selected emission ranges was verified. Overlays of different staining patterns in living cells were directly obtained as snapshots from Leica experimental files. Photographs of fixed cells, showing CFP/YFP costaining were derived by overlay of individual images in Adobe photoshop version 7.0.

FLIP analysis

These experiments were performed using cocultures of HeLa cells that expressed complementary YFP/CFP variants of Fas and FasL. Neighboring cells showing suitable receptor-ligand aggregates were analyzed. In all photo-bleaching experiments the excitation was performed at 514 nm (YFP channel) and the laser energy was constantly maintained at 80% of maximum. The bleached area included most of the cell that expressed the YFP-tagged fusion protein, but excluded receptor-ligand clusters or a small part of the plasma membrane. The analysis took further advantage of the time lapse function, provided by the Leica confocal software. Cells were sequentially imaged in both YFP and CFP channels in 1-min time intervals. Between these times, a maximal excitation applied to the selected bleached areas. Images of each experiment were merged into a single Leica experimental series, as recommended in the software manual. This facilitated the determination of fluorescence intensities in corresponding sections of all merged images. Receptor-ligand clusters, plasma membrane areas and control regions were selected as region of interests (ROIs) and ROI fluorescence was quantified, using an integrated function of the Leica Confocal Software. Importantly, we have manually verified that the selected ROIs corresponded in all cases to the analyzed structures in each single image, because we did occasionally observe movements of the cells or the microscopic stage. The cluster specific YFP fluorescence was obtained by correction for the fluorescence of the corresponding "free" compound in a comparable ROI. Dependent on the morphology of the

clusters and the shape of the ROIs fluorescence of "free" YFP-FasL or Fas-YFP was between 50 and 80% of the fluorescence of the clustered molecules. However, after five bleach cycles the fluorescence intensities of the nonclustered fusion proteins outside the bleach region was regularly below 5% of the fluorescence of clustered molecules. This time point was therefore defined as $t = 0$. In FLIP experiments with nonclustered Fas-YFP and YFP-FasL, the corresponding fluorescence was corrected for the bleach dynamics in the bleach region itself. The corrected fluorescence intensities were normalized against the corrected relative fluorescence intensity obtained after the initial 5 min. Normalized relative fluorescence intensities of corresponding time points of 12–15 FLIP experiments were averaged and plotted over time. To calculate the time ($t_{1/2}$) when 50% of the fluorescence of the YFP fusion proteins was bleached, the curves obtained were linearized by plotting the natural logarithm of the relative fluorescence intensities over time.

Determination of FasL-Fas cluster incidence

5 \times 10⁶ HeLa cells were transfected with 20 μ g of plasmid DNA encoding Fas-YFP or Fas Δ cyt-YFP by electroporation and cultured in 6 well plates, containing one 18 \times 18 cm glass cover slide per well. In parallel, 2 \times 10⁷ HEK293 cells were transiently transfected with CFP-FasL and cultured overnight. The next day, the medium was removed from the 6 well plates and 2 ml of a 4°C cold cell suspension, containing 2 \times 10⁶ transfected HEK293 cells was added into each well. Plates were centrifuged at 1,200 rpm for 5 min at 4°C and the cover glasses were then incubated at 37°C for the indicated times. Cells were fixed and investigated by fluorescence microscopy. A field of \sim 150 \times 150 μ m of was randomly selected. All Fas-YFP or Fas Δ cyt-YFP expressing cells found within this field were analyzed of whether they were in contact with one or more CFP-FasL expressing HEK293 cell. Isolated Fas-YFP or Fas Δ cyt-YFP expressing HeLa cells that were not neighbored or overlaid by CFP-FasL expressing HEK293 cells were excluded from further analysis. Fas-YFP or Fas Δ cyt-YFP expressing HeLa cells that were in apparent contact with CFP-FasL transfected cells were investigated for cluster formation. Clusters were defined by their spot-like or aggregated morphology, as well as by strong codetection of blue and yellow fluorescence. This analysis was continued in equivalent fields until 100 cell pairs were analyzed. The percentage of cell pairs with clusters of Fas-YFP/Fas Δ cyt-YFP and CFP-FasL are indicated. Cluster incidence of Fas-YFP in Jurkat cells was determined by online fluorescence microscopy. HeLa cells expressing CFP-FasL were cultured in Matek glass bottom dishes and overlaid with 5 \times 10⁶ cells of the indicated Jurkat cell line, which had been transfected with 50 μ g Fas-YFP cells by electroporation. After 3 h, all Fas-YFP expressing cells in the dish which were in apparent contact with CFP-FasL expressing HeLa cells were investigated for cluster formation and the cluster incidence was calculated.

Preparation of detergent insoluble raft membrane microdomain fractions

5 \times 10⁷ cells, expressing the proteins of interest were harvested with a rubber policeman, pelleted, resuspended in 200 μ l serum free RPMI medium, and kept on ice. Cells were then lysed in 200 μ l ice-cold Brij-96 lysis buffer [0.1% Brij-96 in TNE (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Pefabloc, 5 mM iodoacetamide; 1 mM Na₃VO₄, 1 mM NaF)]. The lysates were mixed with 80% sucrose in TNE buffer, transferred into an ultra centrifugation tube and overlaid with 2.8 ml 30% sucrose in TNE, followed by 400 μ l TNE. Samples were centrifuged at 50,000 rpm in a TST 60.4 rotor (Sorvall) for 22 h at 4°C. Proteins in the four collected fractions were precipitated by addition of 1.5 ml ice-cold acetone. Precipitates were resolved in SDS sample buffer and analyzed by Western blotting, using the following antibodies: anti-GFP (mAb; Roche); anti-JNK (rabbit pAb; Cell Signaling) and anti-tubulin (NeoMarkers). Based on localization of Lck-GFP (Janes et al., 1999) and palmitylated YFP (Zacharias et al., 2002) the top fraction (1) was identified as detergent insoluble raft membrane microdomain fraction. The bottom fraction (4), which contained the vast majority of total protein was correspondingly identified as detergent soluble. To dissolve lipid rafts, cells were treated with 20 mM of the cholesterol-depleting drug β MCD in serum-free medium at 37°C for 20 min. Cells were then harvested for analysis or analyzed further by online fluorescence microscopy (37°C, 5% CO₂) for up to one more hour.

FACS analysis

To analyze the effects of FasL stimulation on Fas cell surface expression Fas overexpressing HeLa cells were challenged with anti-Flag mAb M2 (1 μ g/ml) cross-linked soluble Flag-FasL (400 ng/ml) for 1 h on ice or at 37°C and analyzed afterwards by FACS. For this purpose cells were stained with anti-Fas-FITC mAb FAB128 (R&D Systems) which does not compete with Flag-FasL for Fas binding for 1 h on ice. To avoid interference of protein resynthe

sis 25 µg/ml CHX was added 30 min before stimulation. Finally cells were analyzed by FACS.

Online supplemental material

Figs. S1 and S2 show that a YFP/CFP tag does not interfere with the functional properties of Fas, FasL, FasΔcyt and FasLΔcyt. Fig. S3 shows colocalization of Fas-CFP and cholera toxin B-rhodamine in anti-cholera toxin B patched cells. Online supplemental material is available at <http://www.jcb.org/cgi/content/full/jcb.200501048/DC1>.

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