SPOTS: signaling protein oligomeric transduction structures are early mediators of death receptor–induced apoptosis at the plasma membrane

Richard M. Siegel,[1,3] Jagan R. Muppidi,[3] Malabika Sarker,[1] Adrian Lobito,[3] Melinda Jen,[1] David Martin,[1] Stephen E. Straus,[2] and Michael J. Lenardo[1]

[1]Laboratory of Immunology and [2]Laboratory of Clinical Infectious Diseases, National Institute of Allergy and Infectious Diseases, and [3]Immunoregulation Unit, Autoimmunity Branch, National Institute of Arthritis and Musculoskeletal and Skin Diseases, National Institutes of Health, Bethesda, MA 20892

Fas (CD95, APO-1, TNFRSF6) is a TNF receptor superfamily member that directly triggers apoptosis and contributes to the maintenance of lymphocyte homeostasis and prevention of autoimmunity. Although FADD and caspase-8 have been identified as key intracellular mediators of Fas signaling, it is not clear how recruitment of these proteins to the Fas death domain leads to activation of caspase-8 in the receptor signaling complex. We have used high-resolution confocal microscopy and live cell imaging to study the sequelae of early events in Fas signaling. These studies have revealed a new stage of Fas signaling in which receptor ligation leads to the formation of surface receptor oligomers that we term signaling protein oligomerization transduction structures (SPOTS). Formation of SPOTS depends on the presence of an intact Fas death domain and FADD but is independent of caspase activity. Analysis of cells expressing Fas mutations from patients with the autoimmune lymphoproliferative syndrome (ALPS) reveals that formation of SPOTS can be disrupted by distinct mechanisms in ALPS.

Introduction

Cell death mediated by the TNF receptor family member Fas (TNFRSF6 and CD95) is required for normal lymphocyte homeostasis and peripheral immune tolerance (Lenardo et al., 1999). In Fas-deficient *lpr/lpr* mice and patients with germ-line dominant-negative Fas mutations and the autoimmune lymphoproliferative syndrome (ALPS), abnormal lymphocytes accumulate and systemic autoimmunity often ensues (Fisher et al., 1995; Siegel et al., 2000a). Ligation of Fas by Fas ligand (FasL) or agonistic antibodies triggers the rapid recruitment of the death domain (DD) of the adaptor protein FADD to the homologous DD of Fas, followed by the recruitment of procaspase-8 and -10 through the interaction of death-effector domains in the amino termini of FADD and the procaspases. The resulting Fas/FADD/caspase complex is termed the death-inducing signaling complex (DISC; Kischkel et al., 1995). Studies of knockout mice and mutant cell lines established that the DISC is essential for Fas apoptosis signaling in lymphocytes (Varfolomeev et al., 1998; Zhang et al., 1998). DISC assembly differs between cell types in ways that influence the efficiency of Fas signaling. In "type I" cell lines and restimulated primary T cells, the DISC forms efficiently, and apoptosis can be induced with bivalent anti-Fas stimuli without additional cross-linking (Scaffidi et al., 1998). This may be due to the constitutive association of Fas in these cells with glycosphingolipid-enriched detergent-resistant membrane microdomains termed lipid rafts (Muppidi and Siegel, 2004). However, in "type II" cell lines and recently activated primary T cells, the DISC is formed inefficiently, hypercrosslinking of Fas is necessary to induce apoptosis, and Fas is not associated with lipid rafts.

Recent work has identified a series of signaling events that follow Fas binding to FasL or agonistic antibodies. In cell lysates, SDS-stable high-molecular weight oligomers of receptors can be seen to form in seconds, followed by maximal recruitment of FADD and caspase-8 within 15 min (Kischkel et al., 1995). Receptor cap structures and internalization of the receptor have been observed 15 min to 1 h after receptor triggering depending on the experimental system (Cremesti et al., 2001; Algeciras-Schimnich et al., 2002). However, cap formation was found to be dependent on caspase activation, and is therefore not a proximal event in receptor signaling. Of greater

The online version of this article includes supplemental material.

Correspondence to Richard M. Siegel: rsiegel@nih.gov

D. Martin's present address is Dept. of Medicine, University of Washington School of Medicine, Seattle, WA 98195.

Abbreviations used in this paper: ALPS, autoimmune lymphoproliferative syndrome; DD, death domain; DISC, death-inducing signaling complex; FasL, Fas ligand; SPOTS, signaling protein oligomerization transduction structures.

The Journal of Cell Biology, Vol. 167, No. 4, November 22, 2004 735–744
http://www.jcb.org/cgi/doi/10.1083/jcb.200406101

interest are events that occur before and are required for procaspase activation.

How procaspase-8 and 10 are activated after recruitment to the cytoplasmic domain of Fas is not known. It is thought that DISC formation promotes the activation of caspase-8. Forced caspase-8 oligomerization, even in the absence of FADD, is sufficient for its enzymatic activation (Martin et al., 1998; Muzio et al., 1998). More recently, it has been shown that in vitro, caspase-8 enzymatic activation can occur even without cleavage of the proenzyme into its active form (Yang et al., 1998; Boatright et al., 2003; Donepudi et al., 2003). We have found that Fas itself can exist in an oligomerized state before ligand binding (Siegel et al., 2000b). This finding predicts that FasL or multivalent antibody binding may trigger signaling through rearrangement of receptor chains or formation of higher order receptor structures.

Here, we examine consequences of Fas signaling with high resolution confocal microscopy. We find that Fas ligation by agonistic antibody or ligand results in the formation of microscopically visible surface receptor microclusters before caspase enzymatic activity. Fas mutations associated with ALPS that impair formation of these structures do not allow full caspase-8 activation and apoptosis induction.

Results

Formation of signaling protein oligomerization transduction structures (SPOTS) is an early event following Fas receptor ligation

To follow the subcellular localization of endogenous Fas during signaling, we treated the lymphoblastoid B cell line SKW6.4, which expresses relatively high levels of Fas, with anti-Fas mAb or FasL at 37°C, and then fixed and stained the cells with a secondary antibody to reveal the pattern of Fas subcellular localization. In these cells, we observed small spotlike foci of staining beginning 15 min after receptor ligation, followed by caplike structures visible after 30 min (Fig. 1). The small foci measured 0.3–0.5 μm in size. As we will show that these foci represent oligomerized receptors and require signaling protein recruitment for their formation, we will refer to them as SPOTS. Interestingly, in the presence of the caspase inhibitor zVAD-fmk, cells with SPOTS were readily seen, but cells with capped Fas were rarely seen. To determine whether or not SPOTS form before internalization of the receptor, we used a double-labeling strategy to specifically mark surface Fas complexes. As can be seen in Fig. 2 A, in SKW6.4 cells treated for 60 min with anti-Fas, Fas complexes were largely internalized. FACS staining experiments confirmed that surface receptor levels were significantly reduced in these cells (Fig. 2 B). However, in the presence of zVAD-fmk, surface-labeled SPOTS predominated, and FACS staining of zVAD-treated cells revealed normal or even increased levels of surface Fas. Thus, formation of SPOTS occurs at the plasma membrane during the same time frame that the Fas signaling complex assembles at the cytoplasmic tail of the receptor.

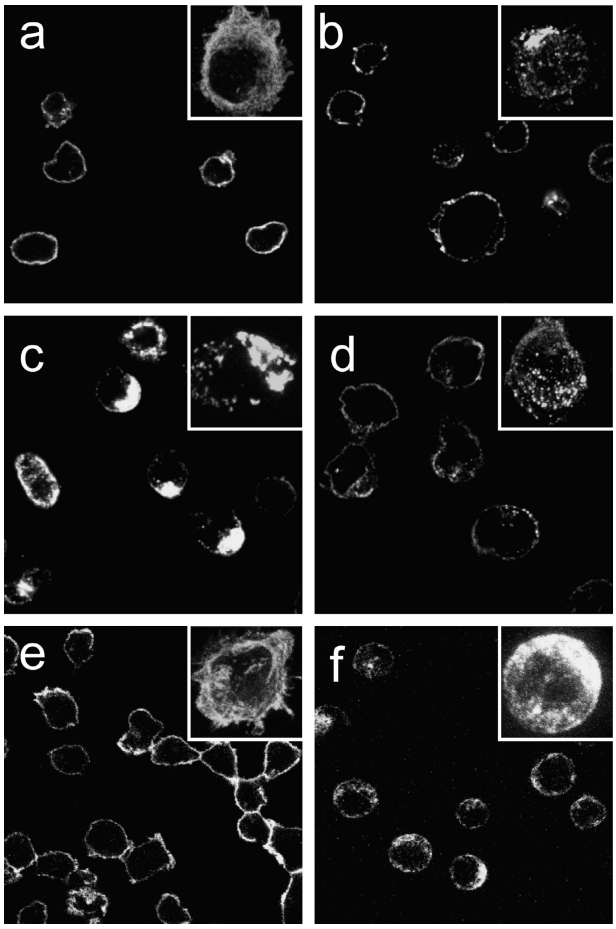

Figure 1. **Distinct caspase-dependent and -independent morphological stages in Fas signaling.** Immunostaining of Fas performed after the following treatments at 37°C: (A) isotype control; (B) 15-min anti-Fas; (C) 30-min anti-Fas; (D) 60-min anti-Fas with 60-min pretreatment with 50 μM zVAD-fmk; (E) 60-min Anti-FLAG control; (F) 60-min FLAG-FasL and anti-FLAG. For each panel, a low-power mid-cell confocal section and a high power reconstruction of a z-stack of a representative cell is shown. Results are representative of three independent experiments.

Lipid raft microdomains can facilitate oligomerization of receptors into larger signaling complexes. Therefore, we investigated the relationship between SPOTS and lipid rafts in different cell types. When lipid rafts were imaged with cholera-toxin B after Fas receptor cross-linking, we observed that lipid raft microdomains coclustered with SPOTS in type I cells (Fig. 3). In type II cells, SPOTS formed without coclustering of the cholera-toxin label, suggesting that lipid rafts remained diffusely distributed on the plasma membrane. This data agrees with previous findings that Fas signaling in type-II cells is independent of lipid rafts (Muppidi and Siegel, 2004).

SPOTS formation requires recruitment of FADD to the Fas DD

To examine if the changes we observed in the subcellular localization of Fas occurred during signaling in live cells, we used confocal microscopy to study the localization of Fas-YFP fusion proteins before and after receptor triggering by FasL or agonistic antibodies. In transiently transfected Cos cells, Fas-

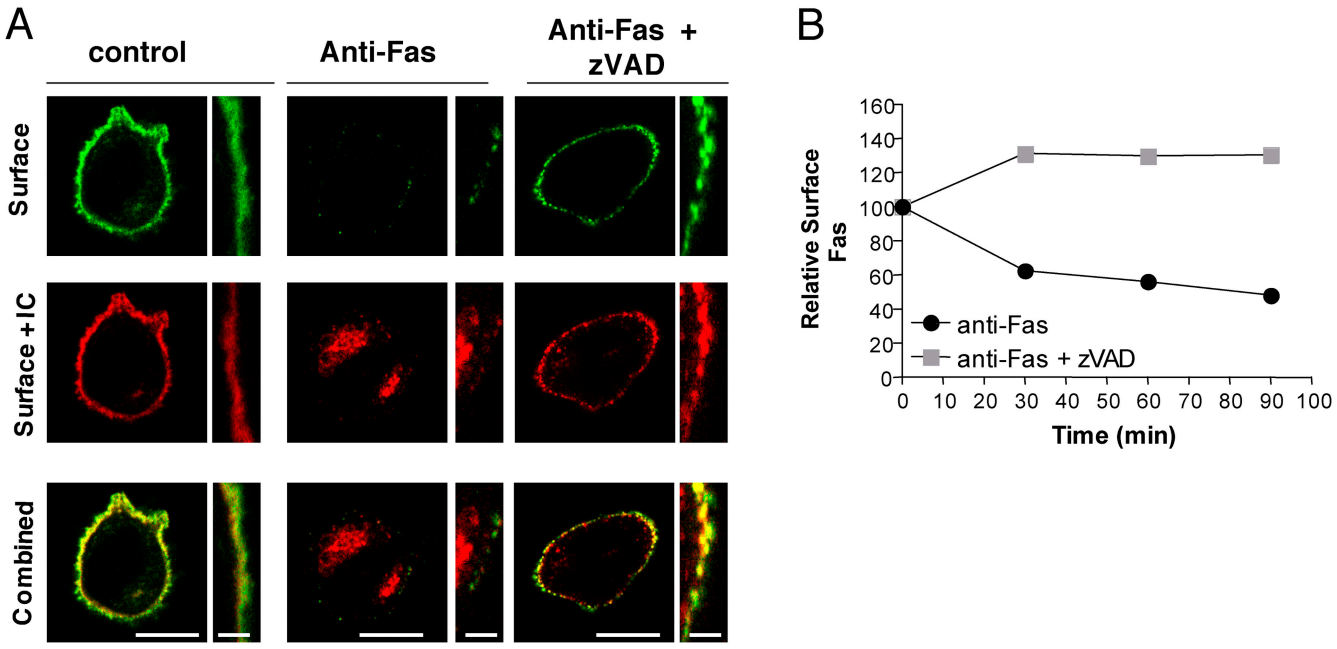

Figure 2. **Capping and internalization but not SPOTS depend on caspase activity.** (A) SKW 6.4 cells were stimulated as shown and then labeled for surface and internalized Fas as described in the Flow cytometric quantitation of Fas surface levels section of Materials and methods. The top panels show Alexa-488 staining of surface Fas in green, the middle panels show surface and intracellular staining by Alexa-594 in red, and the bottom panels show combined fluorescence with colocalized proteins staining in yellow. Separate experiments showed that the two anti-IgG secondary antisera did not cross-block (not depicted). Each panel shows medium and high power magnifications from left to right. Bars: (left) 5 μm; (right) 1 μm. (B) Flow cytometric quantitation of surface Fas expression after similar treatment of SKW 6.4 cells. Numbers are derived from the geometric mean fluorescence normalized a value of 100 for untreated cells.

YFP fusion proteins were distributed diffusely on the plasma membrane in most cells. Some cells exhibited additional intracellular fluorescence in patterns consistent with Golgi complex localization. After addition of agonistic anti-Fas antibody or FasL, intense foci of Fas-YFP fluorescence appeared at the plasma membrane within 8–15 min, peaking at 60 min (Fig. 4 A and Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200406101/DC1). These foci appeared before changes in cell shape or nuclear morphology characteristic of apoptosis. Three-dimensional reconstruction of confocal z-sections of these cells confirmed the surface localization of SPOTS (Fig. 4 A). As with endogenous Fas, caspase inhibition did not block formation of SPOTS. In contrast, deletion of the Fas DD almost completely blocked the formation of SPOTS. We observed quantitatively similar results in SKW6.4 cells transfected with Fas-YFP, including the dependence of SPOTS formation on the Fas DD (Fig. 4 B). FasL was somewhat less efficient at inducing SPOTS despite equivalent maximal levels of apoptosis induced by FasL and anti-Fas mAb (Fig. 4, B and C). These data indicate that Fas SPOTS form in living cells af-

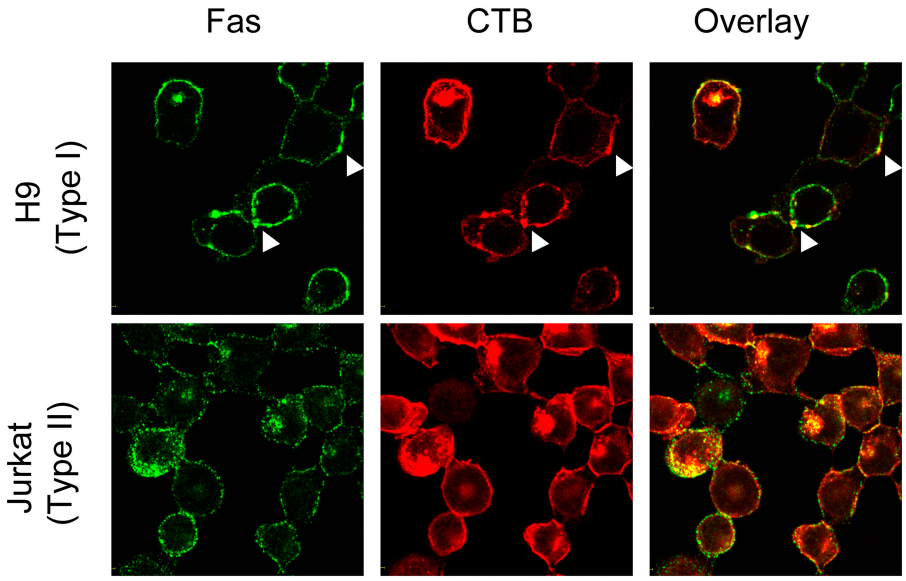

Figure 3. **Differential coclustering of SPOTS with lipid rafts in type I and type II cells.** Two-color fluorescence microscopy of cells treated with anti-Fas in the presence of Alexa-594–labeled cholera toxin B (red) for 30 min and then stained for Fas with anti-IgG3 Alexa-488 (green). Arrowheads mark examples of membrane areas with bright patches of Fas and the CTB staining at the same location.

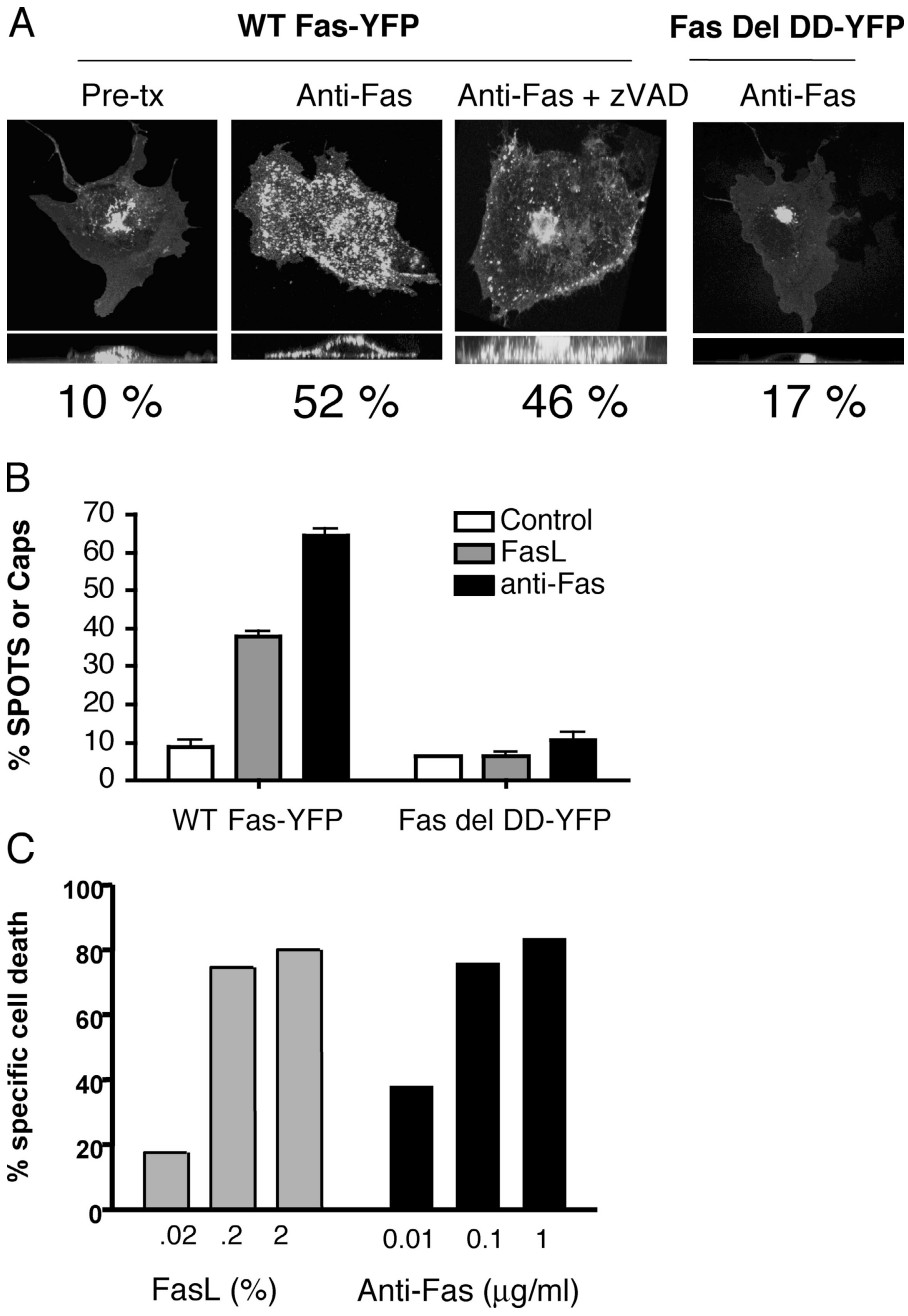

Figure 4. **Redistribution of wild-type and mutant Fas-YFP fusion proteins after anti-Fas treatment.** (A) Cos-7 cells transiently transfected with the indicated Fas-YFP fusion proteins were treated with 1 μg/ml of APO-1 and 0.1 μg/ml of protein A for 30 min at 37°C and z-stack images of live cells were acquired by confocal microscopy. The top panels show vertical maximum intensity projections of the stacks and the bottom panels show a mid-cell section of the same stack rotated 90°. The numbers underneath each panel are the percentage of cells exhibiting receptor SPOTS after 30 min of anti-Fas mAb treatment. (B) Comparison of SPOTS induced by FasL and anti-Fas. SKW6.4 cells were transiently transfected with the indicated Fas-YFP fusion protein constructs and treated with either anti-Fas mAb or FasL-FLAG with anti-FLAG cross-linking as described in the Patients, cell lines, plasmids, and reagents section in Materials and methods. After 60 min at 37°C, live cells were scored for the presence of SPOTS or caps by a blinded observer in duplicate. The numbers are the average ± SEM for each condition. Without anti-FLAG cross-linking <10% SPOTS or caps were observed (not depicted). (C) Cell death induced in SKW6.4 the same preparations of FasL and anti-Fas as in B. Cell death was measured at 6 h by annexin/PI staining.

ter antibody and ligand stimulation and are not simply the result of passive antibody-mediated receptor clustering.

When transfected into Jurkat cells, Fas-YFP also formed SPOTS after receptor ligation, followed by receptor internalization, although with less prominent cap formation. An example of time-lapse images from a typical transfectant is shown in Fig. 5 and Video 2 (available at http://www.jcb.org/cgi/content/full/jcb.200406101/DC1). To determine whether or not FADD and caspase-8 are required for formation of SPOTS, we transfected wild-type Fas-YFP into mutant Jurkat cells that have been shown to lack FADD or caspase-8 and are completely resistant to Fas-induced apoptosis (Juo et al., 1998). As shown in Table I, FADD-deficient Jurkat cells as well as wild-type Jurkat cells transfected with a DD-deficient truncated Fas-YFP were almost completely deficient in SPOTS formation. Caspase-8–deficient Jurkat cells were partially deficient in SPOTS formation. As in other cell types, zVAD-fmk treatment of wild-type cells did not affect SPOTS formation although apoptosis was completely inhibited.

To determine whether receptor internalization was downstream or coincident with formation of SPOTS during signaling in live cells, we developed a flow cytometric assay to measure surface levels of Fas-YFP fusion protein before and after addition of agonistic antibodies. Fas-YFP internalization was slower than endogenous Fas in SKW 6.4, with maximal decrease in surface staining after 4 h of anti-Fas or FasL treatment. Jurkat cells transfected with DD-deleted Fas failed to in-

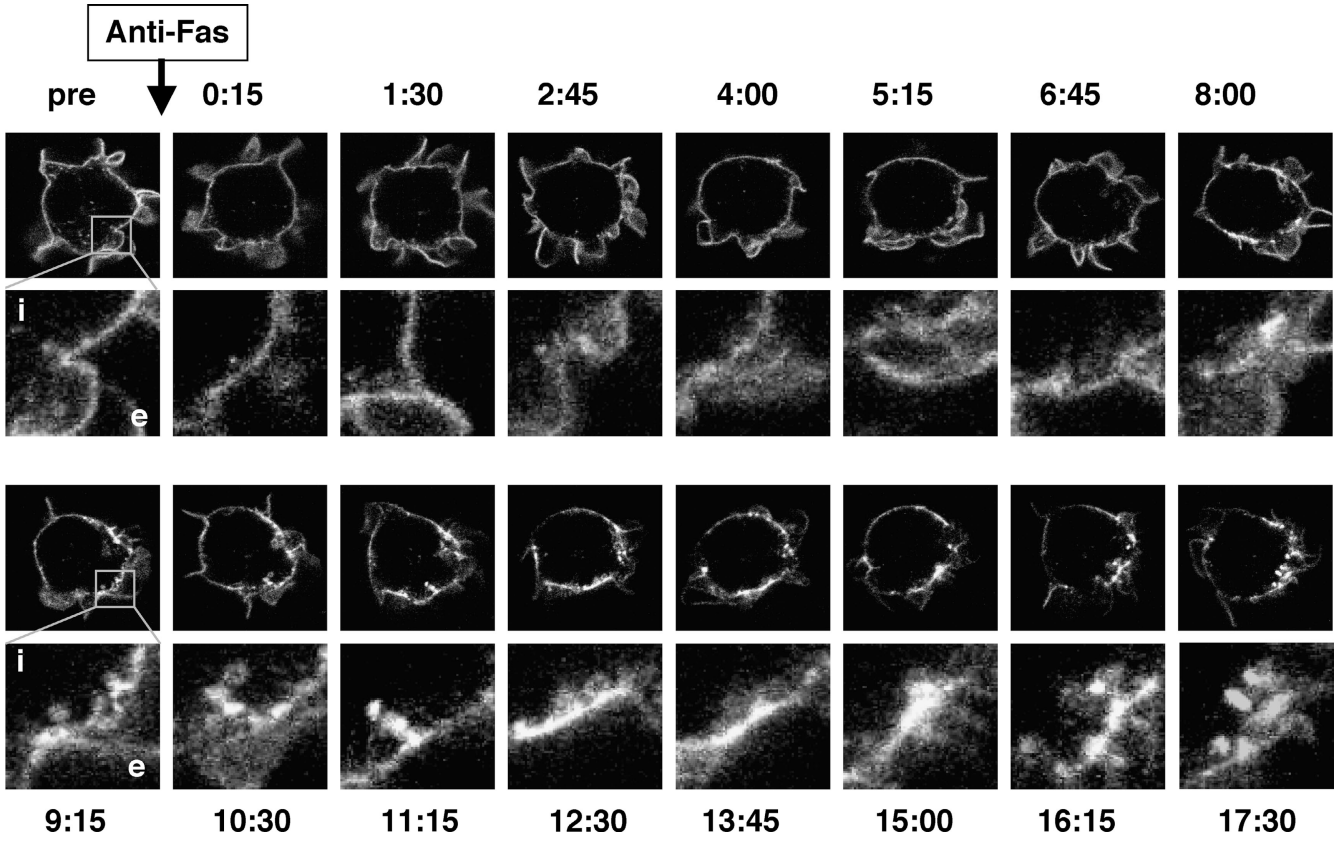

Figure 5. **Kinetics of SPOTS formation and internalization of Fas-YFP fusion proteins.** (A) Selected frames from a confocal time series of Jurkat cells transfected with wild-type Fas-YFP fusion protein and treated with anti-Fas at the indicated time point (minutes:seconds). Midsection confocal images of the whole cell are shown in the top panels, and an enlarged view of a portion of the plasma membrane is shown at each time point with the intracellular (i) and extracellular (e) compartments in the first frame of each row.

ternalize the receptor after anti-Fas or FasL treatment (Fig. 6 A). As expected, FADD and caspase-8–deficient Jurkat cells were almost completely resistant to Fas-induced apoptosis. FADD-deficient Jurkat cells also failed to internalize Fas-YFP after receptor ligation and caspase-8–deficient Jurkat cells had partial inhibition of receptor downmodulation (Table I). Caspase inhibition with zVADfmk also partially blocked Fas-YFP internalization, despite having little effect on formation of SPOTS (Table I and Fig. 6 A). Thus, as with endogenous Fas, downmodulation of Fas-YFP in living cells is downstream of SPOTS formation and dependent on caspase activity. Receptor endocytosis appeared to be clatharin independent because surface downmodulation was not blocked by a dominant-negative dynamin construct (unpublished data).

Table I. **Quantitation of Fas-YFP fusion protein clustering and downmodulation in live Jurkat T cells**

Cell type (Jurkat derivative)	Fas-YFP fusion construct	Treatment	Percentage of SPOTS or caps ± SD	Surface Fas downmodulation (% control)	Fas-induced apoptosis (% control)
WT	WT	Anti-Fas	(100)	(100)	(100)
WT	WT	Anti-Fas + zVAD-fmk	96.4	38.1	<0
WT	Del DD	Anti-Fas	16.1	11.9	3.3
WT	T225K	Anti-Fas	21.1	<0	33
FADD deficient	WT	Anti-Fas	15.7	<0	2.4
Caspase-8 deficient	WT	Anti-Fas	44.3	23.8	5.5
vFLIP line	WT	Anti-Fas	33.4	3.1	3.4
vFLIP control	WT	Anti-Fas	(100)	(100)	(100)
Bcl-x line	WT	Anti-Fas	101.8	57.1	41
Bcl-x control	WT	Anti-Fas	(100)	(100)	(100)

Cells were treated for 60 min with crosslinked anti-Fas APO-1-3 mAb and microscopically scored for receptor clustering with fluorescence microscopy by a blinded observer. Numbers represent the average and SD of at least two independent experiments. Specific Fas downmodulation was calculated from the median channel fluorescence data shown in Fig. 6. Fas-induced apoptosis was calculated from annexin and PI staining and normalized to the percent specific death in the appropriate control cells.

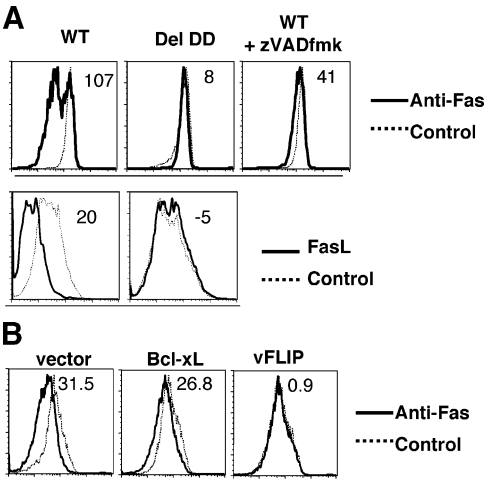

Figure 6. **Dependence of Fas-YFP receptor internalization on the Fas DD and caspase activity.** (A) FACS analysis of Fas-YFP internalization in transfected wild-type Jurkat cells after anti-Fas (top) or FasL (bottom) treatment for 2 h. The dashed histograms represent levels of Fas-YFP surface expression before treatment and the solid lines represent levels after treatment. The numbers are the change in geometric mean channel fluorescence between treated and untreated cells for each transfectant, with positive numbers indicating decreased fluorescence and negative numbers an increase after treatment. Populations were gated on viable cells expressing the Fas-YFP fusion protein. The fusion protein constructs are indicated above each panel. (B) FACS analysis of internalization of WT Fas-YFP fusion proteins in Jurkat clones stably overexpressing Bcl-x and v-FLIP and a control clone transfected with the drug-resistance plasmid alone. Results are shown as in A.

Because caspase inhibitors blocked both apoptosis and receptor downmodulation, it was possible that receptor downmodulation was a consequence of effector caspase activation during apoptosis. To examine this possibility, we used Jurkat cells stably transfected with the antiapoptotic inhibitor Bcl-xL, which blocks mitochondrial amplification of the apoptosis signal and is required in Jurkat cells for efficient Fas-mediated apoptosis. Bcl-xL overexpression does not affect recruitment and activation of caspase-8 at the DISC (Stegh et al., 2002). Fas-YFP in Bcl-xL transfectants was equally able to form SPOTS, and Fas downmodulation was 57% of vector-transfected cells (Table I and Fig. 6 B). In contrast, in a Jurkat line overexpressing the viral FLIP MC159, a proximal blocker of caspase-8 recruitment and activation in the DISC, both SPOTS formation and receptor downmodulation were more severely impaired (Fig. 6 B and Table I). These findings suggest that Fas can be internalized after SPOTS formation without activation of post-mitochondrial effector caspases.

ALPS mutations block formation of SPOTS and caspase-8 activation through distinct mechanisms

Most ALPS-associated Fas mutations in the DD fail to bind to FADD, resulting in dominant inhibition of DISC formation (Martin et al., 1999; Vaishnaw et al., 1999). However, unlike all other previously described Fas DD mutants, the Fas T225K mutation from ALPS kindred #27 retained the ability to interact with FADD in GST pull-down and coimmunoprecipitation assays, although FADD binding was weaker than for wild-type Fas (Fig. 7 A and not depicted). Mutation of the same amino

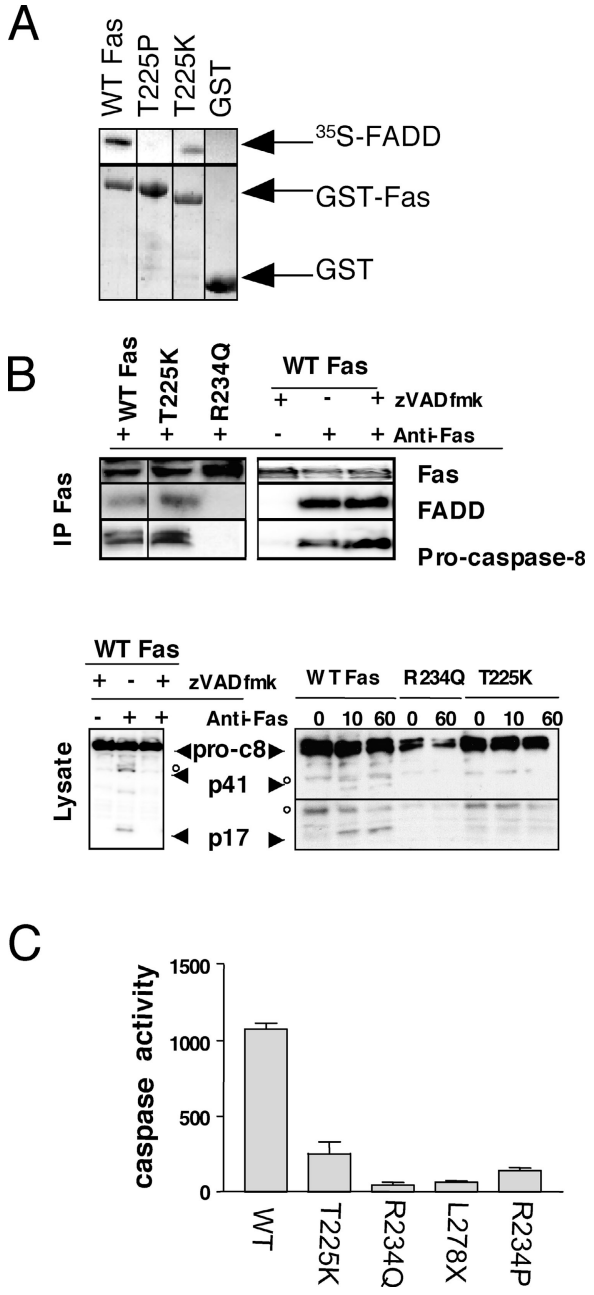

Figure 7. **ALPS-associated Fas mutations block SPOTS formation by distinct mechanisms.** (A) GST pull-down assay with the indicated mutant Fas DD proteins or GST alone, and ^{35}S-labeled FADD, was performed as described previously (Martin et al., 1999). Vertical black lines divide lanes taken from the same blot. (B) Signaling complex formation and caspase-8 processing in EBV lines from selected Fas mutant ALPS patients and zVAD-fmk treated normal donor-derived cells. EBV cell lines were stimulated with 1 μg/ml of APO-1 anti-Fas mAb for 10 min or the times indicated, and the DISC was immunoprecipitated as described previously (Martin et al., 1999). Caspase-8 cleavage in cell lysates are shown in the bottom panel. Arrowheads denote protein fragments of interest and open circles denote background bands. Vertical black lines divide lanes taken from the same blot. (C) DEVDase effector caspase assay of lysates from EBV-transformed cell lines or ALPS patient EBV cell lines heterozygous for the indicated Fas mutations. Cells were treated for 1 h with 1 μg/ml of APO-1 anti-Fas mAb. Assays were performed as described previously (Martin et al., 1999). Values are the fluorescence of treated minus control for each cell line in arbitrary fluorescence units. Error bars represent the SEM for triplicate measurements.

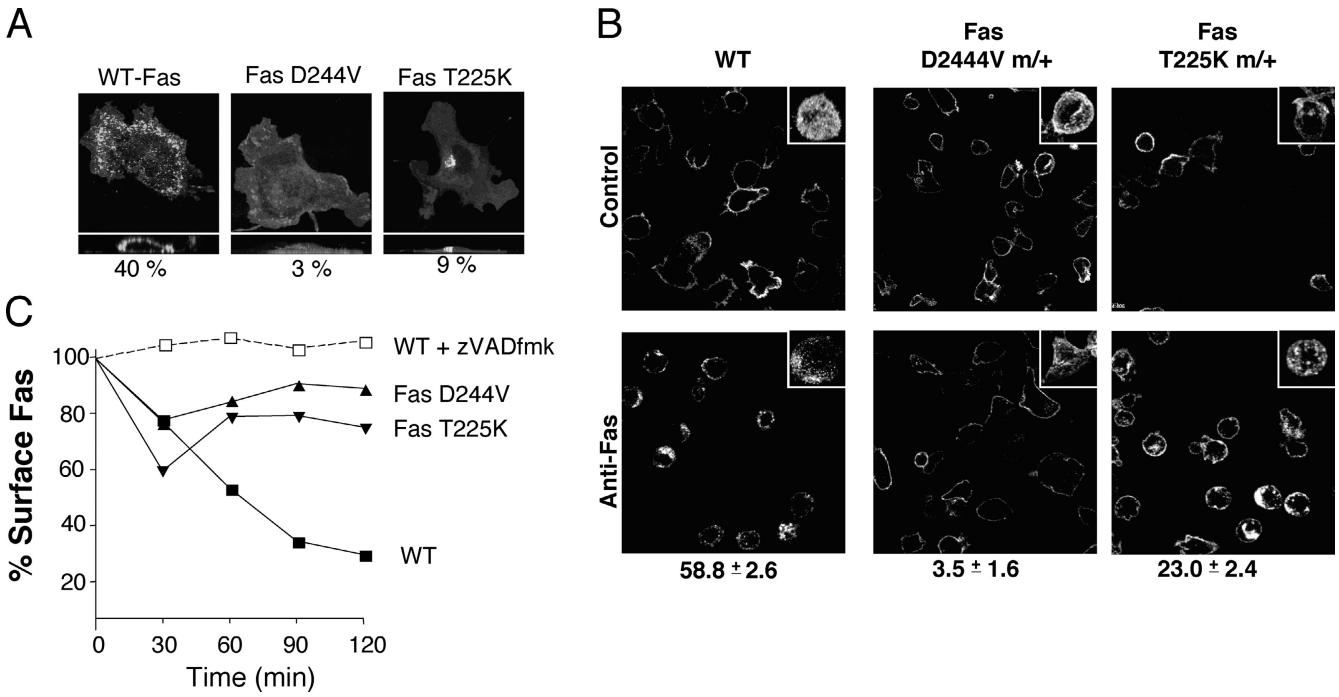

Figure 8. **ALPS Fas mutations and patient cells display defective SPOTS formation and receptor downmodulation.** (A) Cos-7 cells transiently transfected with the indicated Fas-YFP fusion proteins were treated with 1 μg/ml of APO-1 and 0.1 μg/ml of protein A for 30 min at 37°C and z-stack images of live cells were acquired by confocal microscopy and shown as in Fig. 2. (B) EBV-transformed B cell lines from healthy donors or patients with the indicated mutations were treated with anti-Fas for 1 h, stained for Fas as described in Materials and methods, and visually scored for receptor surface clustering or capping, with the total percentage of capped or clustered Fas given below each panel. Mid-cell confocal sections and three-dimensional reconstructions of representative cells are shown for anti-Fas–treated (bottom) or untreated (top) cells. Insets show three-dimensional reconstruction of z-stack images for a representative cell in each condition. The numbers below each panel represent the percentage of cells exhibiting SPOTS or caps after anti-Fas treatment. (C) Quantitation of surface Fas after treatment of the indicated EBV patient-derived cells with anti-Fas and normal cells treated with or without the zVAD-fmk caspase inhibitor.

acid to proline in the T225P mutant from another ALPS family completely abrogated FADD binding and globally perturbed DD folding (Fig. 7 A; Martin et al., 1999). However, the Fas T225K DD retained a normal overall structure (unpublished data). Analysis of DISC formation in patient-derived cell lines harboring the T225K mutant showed normal recruitment of FADD and procaspase-8 after anti-Fas stimulation, whereas cells from ALPS patients harboring the non-FADD binding R234Q mutation or other Fas DD mutations demonstrated severe defects in DISC formation (Fig. 7 B; Martin et al., 1999). Despite normal DISC formation, cells harboring the T225K mutation produced no detectable fully processed caspase-8 in cell lysates even after prolonged Fas stimulation (Fig. 7 B). This pattern was similar to wild-type cells treated with anti-Fas in the presence of zVAD-fmk (Fig. 7 B). Apoptosis, as measured by effector caspase activation, was impaired in cell lines derived from patients heterozygous for the Fas T225K mutation, but was slightly higher than that in cell lines from patients harboring non FADD-binding DD mutations (Fig. 7 C). This finding is consistent with data showing that FasT225K is defective when transfected alone and is a weak dominant-negative inhibitor of apoptosis triggered by wild-type Fas (Jackson et al., 1999).

To correlate these biochemical abnormalities in Fas signaling with the subcellular localization of Fas, we transfected Fas-YFP fusion proteins containing non-FADD binding and the T225K FADD-binding Fas mutations into COS-7 cells and observed the morphology of receptor fluorescence in live cells after anti-Fas antibody treatment. Interestingly, cells transfected with either the non-FADD–binding D244V mutant or the T225K mutant failed to form SPOTS (Fig. 8 A). Similar results were seen with Jurkat cells transfected with FasT225K-YFP (Table I). Immunofluorescence analysis of endogenous Fas in EBV-transformed lymphoblastoid cell lines from ALPS patients bearing these same two mutations revealed an almost complete defect in formation of SPOTS in cells harboring heterozygous FasD244V mutations after treatment with anti-Fas mAb (Fig. 8 B). Cells heterozygous for the T225K Fas mutation exhibited a partial defect in formation of SPOTS as well as defects in receptor downmodulation and cap formation after receptor cross-linking (Fig. 8, B and C), although these defects were not as severe as in cells harboring non FADD-binding Fas mutations. Thus, the defect in caspase-8 processing in the DISC of heterozygous Fas T225K cells specifically impairs FAS SPOTS formation and receptor internalization downstream of FADD recruitment.

Discussion

Fas apoptosis signaling depends on activation of procaspase-8 that is directly recruited to the cytoplasmic tail of the receptor through the adaptor protein FADD. Oligomerization of caspase-8 can activate its proteolytic activity even in its uncleaved pro-enzyme state (Yang et al., 1998; Boatright et al., 2003;

Donepudi et al., 2003). Thus, it is critical to understand how ligation of Fas and other death receptors activate this apical enzyme in the receptor-induced apoptosis pathway. Our data suggest that formation of SPOTS may promote caspase-8 activation in receptor signaling complexes through aggregation of receptors and associated signaling molecules into large microscopically visible surface clusters. Given the surface area of 23 nm^2 for the structurally related TNF/TNFR complex (Banner et al., 1993), a large number of trimers could occupy a circular membrane patch with a diameter of 300 nm, the size of the smallest clusters we were able to visualize by confocal microscopy. We previously reported that Fas and other TNF receptor family members can self-associate at the molecular level in preassociated complexes before ligand binding (Chan et al., 2000; Siegel et al., 2000b). These molecular complexes are likely to contain fewer receptor chains than SPOTS, as the same cells exhibiting receptor self-association by techniques such as coimmunoprecipitation and fluorescence resonance energy transfer in the absence of ligand exhibited a diffuse pattern of receptor expression on the membrane with very few SPOTS (<10% of cells).

Formation of Fas SPOTS is dependent on recruitment of the adaptor protein FADD to the DD in the cytoplasmic tail of the Fas receptor. Cells harboring Fas mutations that fail to recruit FADD, or FADD-deficient cells, were completely unable to form SPOTS, demonstrating a critical requirement for FADD in this process. In other work, we have found that FADD self-association through a conserved short peptide motif is required for Fas signaling and the formation of SPOTS (unpublished data). Together, these results define a role for SPOTS in amplifying Fas-mediated signals through homophilic adaptor protein interactions. Whether or not other TNF-family receptors similarly form SPOTS during signaling is not known. Interestingly, in contrast to Fas, which directly recruits FADD at the plasma membrane, the DISC of TNFR1 has been recently reported to assemble only after receptor internalization, illustrating diversity of signaling mechanisms within the death receptor family (Schneider-Brachert et al., 2004). Unlike other previously described Fas mutations, the Fas T225K mutation was able to directly interact with FADD yet did not support formation of SPOTS. The non-FADD–binding ALPS-associated Fas mutations block SPOTS formation indirectly by preventing efficient FADD recruitment to the DISC. Residue Thr 225 of the Fas DD is located on the surface between the α-1 and α-2 helices, away from the Fas/FADD interface localized to α-3. As assessed by NMR, the overall folding of the Fas T225K DD was preserved, whereas the T225P mutation significantly disrupted folding of the DD and abrogated FADD binding (Martin et al., 1999). This result suggests that the T225 residue is not itself directly involved in FADD binding. T225 could be involved in Fas–Fas interactions, but we have found that self-interaction through the DD of T225K mutant Fas is maintained (unpublished data). Instead, lower affinity for FADD or altered quaternary structure of signaling complexes containing the Fas T225K mutation may not allow for FADD lateral interactions, precluding the formation of SPOTS and caspase-8 processing. Although in vitro the caspase-8 proenzyme can become enzymatically active at high concentrations (Boatright et al., 2003), the data with the T225K mutation suggest that the function of SPOTS is to allow caspase-8 to concentrate at the plasma membrane at sufficiently high levels to achieve full enzymatic activation in vivo.

In contrast to deficiencies of signaling proteins, we found that caspase inhibitors had only modest effects on SPOTS but almost completely blocked Fas capping and internalization. A previous paper noted that zVAD-fmk blocked ligand-induced receptor clustering (Algeciras-Schimnich et al., 2002). However, the morphology of what was termed clustering in that paper more closely resembles what we describe here as capping and is thus a later step in signaling than SPOTS. Our double-labeling experiments show that zVAD-fmk treatment allows SPOTS formation but prevents receptor internalization. Thus it is likely that SPOTS result from protein–protein interactions and other cellular events that are independent of caspase enzymatic activity. The formation of SDS-stable microaggregates of Fas have also been reported to be an almost instantaneous result of receptor ligation and are independent of caspase activity (Algeciras-Schimnich et al., 2002). Because these aggregates can be observed in as little as 1 s after addition of anti-Fas in contrast to the minimum of 5–10 min for SPOTS formation in vivo it appears SPOTS are a later event than SDS-stable microaggregates.

Fas-induced receptor capping has also been associated with ceramide production (Grassme et al., 2001). Because ceramide production has been shown to be blocked by caspase inhibition (Cock et al., 1998), it is likely that ceramide production is downstream of SPOTS formation in the Fas signaling pathway. The role that receptor internalization may play in an irreversible process such as apoptosis is not clear. However, it should be noted that other biological outcomes, such as proliferation and lymphocyte activation, have also been associated with death receptor signaling, and receptor internalization may be a way to terminate these signals. What effect caspase inhibition has on the internalization of other receptor classes is not known.

Our findings with the Fas T225K mutation are the first description of a pathogenic receptor mutation that specifically impairs receptor aggregation during signaling. Additional mutations in Fas or other TNFR-family receptors may also use this mechanism of disease pathogenesis. As SPOTS appear to be a proximal manifestation of receptor signaling that are required for signaling to proceed, inhibiting formation of SPOTS may be a therapeutic target for strategies designed to modulate signaling by the many clinically important members of the TNF-receptor superfamily.

Materials and methods

Patients, cell lines, plasmids, and reagents

ALPS patients and their specimens were studied with informed consent according to research protocols approved by National Institutes of Health (NIH) Institutional Review Boards. They and their specific Fas mutations were described previously (Jackson et al., 1999). EBV transformed cell lines from ALPS patients' peripheral blood B cells were derived by R. Fischer (National Human Genome Research Institute, NIH, Bethesda, MD), as described previously (Jackson et al., 1999). The cell lines Jurkat

A3 (wild-type), Jurkat I9.2 (caspase-8 deficient), and Jurkat I2.1 (FADD deficient) were obtained from American Type Culture Collection. Cos-7 cells were a gift from A. Kenworthy (National Institute of Child Health and Human Development, NIH, Bethesda, MD). HA-Fas-GFP and YFP fusion protein vectors were constructed as previously described (Siegel et al., 2000b), using the pEGFPN1 and pEYFPN1 vectors (CLONTECH Laboratories, Inc.) and vectors obtained from R. Tsien (University of California, San Diego, San Diego, CA). Mutations were created by site-directed mutagenesis (quickchange protocol; Stratagene) and confirmed by sequencing. The MC159 and bcl-x–transfected Jurkat lines were a gift from D. Bolton (NIH, Bethesda, MD). The FLAG-FasL plasmid was a gift from G. Screaton (Oxford University, Oxford, UK), and protein was made by transfecting 293T cells in 20-cm cell culture dishes. After an overnight incubation, the culture medium was changed to CHO-S-SFM II medium (Invitrogen). 4 d later, the supernatant from the transfected cells was harvested, filtered to remove any debris, and aliquoted for storage at −80°C. Cos cells transfected in coverslip dishes were transfected with 1 μg of each plasmid using the FuGene transfection reagent (Roche Molecular Biochemicals). 1–2 × 10^7 Jurkat cells were transfected using a BTX electroporator as described previously (Martin et al., 1999). Anti-Fas APO-1-3 was purchased from Kamiya Biochemicals, and Alexa-conjugated secondary antibodies were purchased from Molecular Probes. For cell staining, all mAbs and secondary reagents were used at 1 μg/ml. ZVAD-fmk was purchased from Enzyme Systems Products.

Immunofluorescence and imaging

For live cell imaging, transfected Cos cells were grown on coverslip chambers (Lab-Tek), and Jurkat, H9, or SKW 6.4 cells were added to coverslips coated with poly-L-lysine immediately before imaging. Samples were heated to 37° and APO-1-3 anti-Fas mAb (1 μg/ml with 0.1 μg/ml of protein-A) or FLAG-FasL (1:20 dilution of culture supernatant with 1 μg/ml anti-FLAG) was added directly to the cells. For staining of fixed cells, cells were treated with antibodies or FasL at 37° for the indicated amounts of time and adhered to poly-L-lysine–coated coverslips for the last 5–15 min of culture. Cells were then fixed in 100% methanol at −20° for 7 min, followed by washing and staining with the indicated antibodies and secondary reagents in PBS/0.01% Tween-20/0.5% BSA (IFA buffer). Quantitation of receptor clustering was performed by a blinded observer and at least two counts of >100 cells each made for every condition in an experiment. Cells with either SPOTS or caps were counted as clustered. Only those cells with homogeneous membrane staining were counted as nonclustered. For simultaneous surface and intracellular staining of Fas, SKW6.4 cells were treated with 1 μg/ml of APO-1 anti-Fas for 1 h at 37°C. Anti-IgG3 Alexa-488 was added for 30 min on ice in FACS buffer to visualize residual surface Fas. Cells were fixed and permeabilized and anti-IgG3 Alexa-594 was added to visualize both surface and intracellular Fas. Images were acquired on a confocal microscope (TCS-NT [Leica]; LSM 510 [Carl Zeiss MicroImaging, Inc.]) with 63 or 100× objectives. Images were processed with IMARIS software (Bitplane, A.G.).

Flow cytometric quantitation of Fas surface levels

Jurkat cells were transiently transfected with wild-type and mutant HA-Fas-YFP fusion protein vectors as described previously (Martin et al., 1999). Cells were treated with 1 μg/ml of APO-1 anti-Fas mAb plus 0.1 μg/ml of protein A or FLAG-FasL at 1:100 dilution and 1 μg/ml anti-FLAG mAb at 37°C for the indicated times. Cells were stained for surface Fas on ice with 1 μg/ml anti-HA biotin and 1 μg/ml of avidin Alexa-633 (Molecular Probes). Propidium Iodide negative viable cells positive for transfected Fas-YFP were gated and Alexa-633 Fas surface staining was quantitated. Relative fluorescence was calculated from the ratios of mean channel fluorescence of the YFP positive cells. For quantitation of endogenous surface Fas levels, cells were treated with 1 μg/ml of APO-1-3 for the indicated amounts of time at 37°C, and then stained with anti-mouse Alexa-488 on ice before fixation. Control cells were treated with anti-Fas for equal amounts of time at 4°C for establishing the baseline levels of surface Fas. Surface Fas levels on viable cells were quantitated as for transfected cells.

Online supplemental material

Time-lapse confocal microscopy videos illustrating formation of Fas SPOTS in Fas-YFP–transfected Cos-7 and Jurkat cells are available online. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200406101/DC1.

We would like to thank Owen Schwartz of the National Institute of Allergy and Infectious Diseases light imaging facility and Kristien Zaal of the National Institute of Arthritis and Musculoskeletal and Skin Diseases light imaging facility for their technical assistance. We would also like to thank Joie Davis and Roxanne Fischer for patient enrollment and preparation of samples, Janet Dale for patient care and coordination, and Jennifer Puck for advice and comments on the manuscript. We would also like to thank Diane Bolton and Gavin Screaton for providing valuable reagents for this study.

Submitted: 16 June 2004
Accepted: 17 September 2004

References

Algeciras-Schimnich, A., L. Shen, B.C. Barnhart, A.E. Murmann, J.K. Burkhardt, and M.E. Peter. 2002. Molecular ordering of the initial signaling events of CD95. *Mol. Cell. Biol.* 22:207–220.

Banner, D.W., A. D'Arcy, W. Janes, R. Gentz, H.J. Schoenfeld, C. Broger, H. Loetscher, and W. Lesslauer. 1993. Crystal structure of the soluble human 55 kd TNF receptor-human TNF beta complex: implications for TNF receptor activation. *Cell.* 73:431–445.

Boatright, K.M., M. Renatus, F.L. Scott, S. Sperandio, H. Shin, I.M. Pedersen, J.E. Ricci, W.A. Edris, D.P. Sutherlin, D.R. Green, and G.S. Salvesen. 2003. A unified model for apical caspase activation. *Mol. Cell.* 11:529–541.

Chan, F.K., H.J. Chun, L. Zheng, R.M. Siegel, K.L. Bui, and M.J. Lenardo. 2000. A domain in TNF receptors that mediates ligand-independent receptor assembly and signaling. *Science.* 288:2351–2354.

Cock, J.G., A.D. Tepper, E. de Vries, W.J. van Blitterswijk, and J. Borst. 1998. CD95 (Fas/APO-1) induces ceramide formation and apoptosis in the absence of a functional acid sphingomyelinase. *J. Biol. Chem.* 273:7560–7565.

Cremesti, A., F. Paris, H. Grassme, N. Holler, J. Tschopp, Z. Fuks, E. Gulbins, and R. Kolesnick. 2001. Ceramide enables fas to cap and kill. *J. Biol. Chem.* 276:23954–23961.

Donepudi, M., A.M. Sweeney, C. Briand, and M.G. Grutter. 2003. Insights into the regulatory mechanism for caspase-8 activation. *Mol. Cell.* 11:543–549.

Fisher, G.H., F.J. Rosenberg, S.E. Straus, J.K. Dale, L.A. Middleton, A.Y. Lin, W. Strober, M.J. Lenardo, and J.M. Puck. 1995. Dominant interfering Fas gene mutations impair apoptosis in a human autoimmune lymphoproliferative syndrome. *Cell.* 81:935–946.

Grassme, H., H. Schwarz, and E. Gulbins. 2001. Molecular mechanisms of ceramide-mediated CD95 clustering. *Biochem. Biophys. Res. Commun.* 284:1016–1030.

Jackson, C., R.E. Fischer, A.P. Hsu, S.M. Anderson, Y. Choi, J. Wang, J.K. Dale, T.A. Fleisher, L.A. Middelton, M.C. Sneller, et al. 1999. Autoimmune lymphoproliferative syndrome with defective Fas: genotype influences penetrance. *Am. J. Hum. Genet.* 64:1002–1014.

Juo, P., C.J. Kuo, J. Yuan, and J. Blenis. 1998. Essential requirement for caspase-8/FLICE in the initiation of the Fas-induced apoptotic cascade. *Curr. Biol.* 8:1001–1008.

Kischkel, F.C., S. Hellbardt, I. Behrmann, M. Germer, M. Pawlita, P.H. Krammer, and M.E. Peter. 1995. Cytotoxicity-dependent APO-1 (Fas/CD95)-associated proteins form a death-inducing signaling complex (DISC) with the receptor. *EMBO J.* 14:5579–5588.

Lenardo, M., K.M. Chan, F. Hornung, H. McFarland, R. Siegel, J. Wang, and L. Zheng. 1999. Mature T lymphocyte apoptosis—immune regulation in a dynamic and unpredictable antigenic environment. *Annu. Rev. Immunol.* 17:221–253.

Martin, D.A., R.M. Siegel, L. Zheng, and M.J. Lenardo. 1998. Membrane oligomerization and cleavage activates the caspase-8 (FLICE/MACHalpha1) death signal. *J. Biol. Chem.* 273:4345–4349.

Martin, D.A., L. Zheng, R.M. Siegel, B. Huang, G.H. Fisher, J. Wang, C.E. Jackson, J.M. Puck, J. Dale, S.E. Straus, et al. 1999. Defective CD95/APO-1/Fas signal complex formation in the human autoimmune lymphoproliferative syndrome, type Ia. *Proc. Natl. Acad. Sci. USA.* 96: 4552–4557.

Muppidi, J.R., and R.M. Siegel. 2004. Ligand-independent redistribution of Fas (CD95) into lipid rafts mediates clonotypic T cell death. *Nat. Immunol.* 5:182–189.

Muzio, M., B.R. Stockwell, H.R. Stennicke, G.S. Salvesen, and V.M. Dixit. 1998. An induced proximity model for caspase-8 activation. *J. Biol. Chem.* 273:2926–2930.

Scaffidi, C., S. Fulda, A. Srinivasan, C. Friesen, F. Li, K.J. Tomaselli, K.M. Debatin, P.H. Krammer, and M.E. Peter. 1998. Two CD95 (APO-1/Fas) signaling pathways. *EMBO J.* 17:1675–1687.

Schneider-Brachert, W., V. Tchikov, J. Neumeyer, M. Jakob, S. Winoto-Morbach, J. Held-Feindt, M. Heinrich, O. Merkel, M. Ehrenschwender, D.

Adam, et al. 2004. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. *Immunity*. 21:415–428.

Siegel, R.M., F.K. Chan, H.J. Chun, and M.J. Lenardo. 2000a. The multifaceted role of Fas signaling in immune cell homeostasis and autoimmunity. *Nat. Immunol.* 1:469–474.

Siegel, R.M., J.K. Frederiksen, D.A. Zacharias, F.K. Chan, M. Johnson, D. Lynch, R.Y. Tsien, and M.J. Lenardo. 2000b. Fas preassociation required for apoptosis signaling and dominant inhibition by pathogenic mutations. *Science.* 288:2354–2357.

Stegh, A.H., B.C. Barnhart, J. Volkland, A. Algeciras-Schimnich, N. Ke, J.C. Reed, and M.E. Peter. 2002. Inactivation of caspase-8 on mitochondria of Bcl-xL-expressing MCF7-Fas cells: role for the bifunctional apoptosis regulator protein. *J. Biol. Chem.* 277:4351–4360.

Vaishnaw, A.K., J.R. Orlinick, J.L. Chu, P.H. Krammer, M.V. Chao, and K.B. Elkon. 1999. The molecular basis for apoptotic defects in patients with CD95 (Fas/Apo-1) mutations. *J. Clin. Invest.* 103:355–363. (published erratum appears in *J. Clin. Invest.* 1999. 103:1099).

Varfolomeev, E.E., M. Schuchmann, V. Luria, N. Chiannilkulchai, J.S. Beckmann, I.L. Mett, D. Rebrikov, V.M. Brodianski, O.C. Kemper, O. Kollet, et al. 1998. Targeted disruption of the mouse Caspase 8 gene ablates cell death induction by the TNF receptors, Fas/Apo1, and DR3 and is lethal prenatally. *Immunity.* 9:267–276.

Yang, X., H.Y. Chang, and D. Baltimore. 1998. Autoproteolytic activation of pro-caspases by oligomerization. *Mol. Cell.* 1:319–325.

Zhang, J., D. Cado, A. Chen, N.H. Kabra, and A. Winoto. 1998. Fas-mediated apoptosis and activation-induced T-cell proliferation are defective in mice lacking FADD/Mort1. *Nature.* 392:296–300.