Fas Death Receptor Enhances Endocytic Membrane Traffic Converging into the Golgi Region

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The death receptor Fas/CD95 initiates apoptosis by engaging diverse cellular organelles including endosomes. The link between Fas signaling and membrane traffic has remained unclear, in part because it may differ in diverse cell types. After a systematic investigation of all known pathways of endocytosis, we have clarified that Fas activation opens clathrin-independent portals in mature T cells. These portals drive rapid internalization of surface proteins such as CD59 and depend upon actin-regulating Rho GTPases, especially CDC42. Fas-enhanced membrane traffic invariably produces an accumulation of endocytic membranes around the Golgi apparatus, in which recycling endosomes concentrate. This peri-Golgi polarization has been documented by colocalization analysis of various membrane markers and applies also to active caspases associated with internalized receptor complexes. Hence, T lymphocytes show a diversion in the traffic of endocytic membranes after Fas stimulation that seems to resemble the polarization of membrane traffic after their activation.

INTRODUCTION

Death receptors mediate physiological apoptosis via the “extrinsic” pathway, so named because it follows receptor ligation with cell extrinsic proteins such as tumor necrosis factor- α and Fas-ligand (FasL) (CD95L). The extrinsic pathway has been traditionally envisaged as a linear sequence of events emanating from the complex formed by ligated receptors with adaptor proteins and associated enzymes (Peter and Kramer, 2003). However, it has been increasingly appreciated that death receptors such as Fas/CD95 undergo rapid internalization after triggering (Algeciras-Schimmich *et al.*, 2002; Eramo *et al.*, 2004; Lee *et al.*, 2006), in a process that is essentially equivalent to the well known internalization of other surface receptors such as that for epidermal growth factor (Di Fiore and De Camilli, 2001). The complexes formed by activated death receptors seem to maintain signaling after internalization, in part using the early traffic of endocytic vesicles as a platform for intracellular activation of caspases (Lee *et al.*, 2006; cf. Di Fiore and De Camilli, 2001). This situation seems to apply, in particular, to type I cells,

which do not require mitochondria for caspase activation (Peter and Kramer, 2003).

Most cells of our body require mitochondria for cell execution along the extrinsic pathway and are usually defined of type II (Green *et al.*, 2003; Peter and Kramer, 2003). They include mature T lymphocytes and related lines such as Jurkat, which show distinctive differences from type I cells in trafficking internalized Fas/CD95 (Algeciras-Schimmich *et al.*, 2002; Eramo *et al.*, 2004; Siegel *et al.*, 2004; Lee *et al.*, 2006). In these cells, Fas stimulation increases the uptake of the membrane probe *N*-(3-triethylammonium propyl)-4-(dibutylamino)styrylpyridinium dibromide (Kawasaki *et al.*, 2000; Matarrese *et al.*, 2008) and of pynocytic markers (Kenis *et al.*, 2004; Matarrese *et al.*, 2008). In addition, it induces an unusual intermix of endosomes with mitochondria and other organelles (Ouasti *et al.*, 2007; Matarrese *et al.*, 2008).

The dynamics of endocytic organelles forms part of membrane traffic, which follows diverse portals of endocytosis (Conner and Schmid, 2003; Mayor and Pagano, 2007). After Fas triggering, the clathrin-dependent pathway of endocytosis is likely to be stimulated early to favor receptor internalization (Lee *et al.*, 2006). However, Fas signaling also promotes a progressive block of clathrin-dependent endocytosis via caspase-mediated cleavage of components of this pathway (Austin *et al.*, 2006). Hence, it has remained unclear to what extent Fas-enhanced endocytosis may contribute to the propagation of death signaling (Siegel *et al.*, 2004; Austin *et al.*, 2006; Reihner and Häussinger, 2008; Chaigne-Delalande *et al.*, 2008). Moreover, the specific portal(s) engaged by Fas signaling have not been identified.

We have noted an intriguing similarity between the Fas-induced changes in membrane traffic and those previously documented in the activation of T cells. Fas signaling en-

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Abbreviations used: APC, allophycocyanin; HPA, *Helix pomatia* agglutinin; Rho-IETD-bis, rhodamine110carbonyl-Ile-Glu-Thr-Asp-bisamide; PE, phycoerythrin; RB, modified Ringer buffer; z-VAD, *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone; WGA, wheat germ agglutinin.

Table 1. Portals and systems of endocytosis in T cells

Endocytosis portal or system	Tools/markers	Tested here and references
Phagocytosis Macropinocytosis Clathrin dependent	Congo-red yeast Sulforhodamine 101 Transferrin, CD81	Supplemental Figure S1A Supplemental Figure S1C, Kenis <i>et al.</i> (2004) Figures 1C, 4D, and Supplemental Figure S4A Austin <i>et al.</i> (2006); Lee <i>et al.</i> (2006)
Clathrin and caveolin independent, Rho GTPase dependent	Dextran, CtxB, GPI anchored proteins like CD59	Figures 1, 2, 3, 4, 5, 7, and 8 and Supplemental Figures S1, S2, and S3 Kawasaki <i>et al.</i> (2000); Kenis <i>et al.</i> (2004); Matarrese <i>et al.</i> (2008)
Microparticles/ectosomes	CD59, DioC18	Figure 5D and Supplemental S3A; data not shown Elward <i>et al.</i> (2005)
Trogocytosis	Diverse membrane stains	Luchetti and Degli Esposti, unpublished Davis and Sowinski (2008)

hances exocytosis after the initial wave of increased endocytosis (Ouasti *et al.*, 2007; Reihner and Häussinger, 2008), whereas T cell activation increases both endocytosis and exocytosis, which become polarized at different sides of the cell (Krummel and Macara, 2006). Given this similarity and the complexity of the diverse routes of membrane traffic, we undertook a systematic study of every endocytic system known to be present in T cells (Table 1). We have clarified that Fas enhances Rho GTPase-dependent routes polarizing membranes toward the Golgi region.

MATERIALS AND METHODS

Reagents

Recombinant FasL was purchased from Apotech/Alexis (Lausanne, Switzerland). Fluorescent probes were purchased from Invitrogen (Carlsbad, CA). Lyophilized *Helix pomatia* agglutinin (HPA) (Sigma-Aldrich, St. Louis, MO) was fluorescently conjugated with Alexa350 (blue HPA) by using a kit from Invitrogen. The assay kit for CDC42 was from Millipore (Billerica, MA), whereas antibodies were purchased from the following sources: fluorescein isothiocyanate (FITC)- and allophycocyanin-conjugated anti-CD81 (JS-81), monoclonals for GM130 and Fas/CD95 (DX2) from BD Biosciences (Oxford, United Kingdom), FITC-conjugated anti-CD59 (MEM43) from Invitrogen, phycoerythrin (PE)/Cy5-conjugated anti-CD59 from Leinco Technologies (St. Louis, MO), and FITC-conjugated anti-CD43 (MEM-59) and anti-lysosome associated membrane protein-1 from Santa Cruz Biotechnology (Santa Cruz, CA). *Clostridium difficile* toxin B was obtained from Calbiochem (San Diego, CA), *N*-benzylloxycarbonyl-Val-Ala-Asp-fluoromethylketone (*z*-VAD) was from Alexis Laboratories (San Diego, CA), and other reagents were from Invitrogen and Fisher (Loughborough, United Kingdom). Secramine A was a generous gift from the Kirchhausen laboratory (Harvard Medical School, Boston, MA; Pelish *et al.*, 2006).

Cell Culture

Different batches of the of human acute T cell leukemia line Jurkat J6.1 were obtained from European Tissue Collection (Salisbury, United Kingdom). Likewise the related CEM line and a caspase-8-deficient line (provided by Dr. M. McFairlane, MRC Toxicology Unit, Leicester, United Kingdom), cells were cultured in RPMI 1640 medium supplemented with 10% fetal calf serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin in a humidified atmosphere with 5% (vol/vol) CO₂ at 37°C.

Immunocytochemistry

Cells resuspended in modified Ringer buffer (RB; 145 mM NaCl, 4.5 mM KCl, 2 mM MgCl₂, 1 mM CaCl₂, 5 mM K-HEPES, pH 7.4, and 10 mM glucose) were loaded with diverse fluorescent probes for 20–30 min, washed, and incubated at $\sim 2 \times 10^6$ /ml with recombinant FasL or CH-11 (routinely at a final concentration of 0.5 μ g/ml) before plating onto coverslips coated with polylysine. After an adhesion period of 10–15 min at 37°C, cells were transferred on ice for 5 min (to reduce cellular movement) and then washed with phosphate-buffered saline (PBS) twice before fixation with 4% (wt/vol) paraformaldehyde in PBS. For immunodetection of internal proteins, cells were permeabilized with 0.5% Triton X-100 or 0.1% saponin, blocked with appropriate serum, incubated with monoclonal antibodies for 30–60 min, washed again, and then incubated for 45–60 min with secondary anti-mouse antibodies conjugated with AlexaFluor 488 or Rhodamine X (Ouasti *et al.*, 2007). For

surface staining, fixed cells were treated with diluted solutions of fluorescently-labeled monoclonal antibodies (optimized for each individual application) containing bovine serum albumin to minimize background binding (Naslavsky *et al.*, 2004). Nonspecific fluorescence staining was evaluated using corresponding isotypic immunoglobulin-conjugates (Elward *et al.*, 2005).

Fluorescence and Time-Lapse Microscopy

Fluorescence imaging was carried out in wide-field conventional microscopes (either Zeiss [Carl Zeiss, Jena, Germany] or Olympus [Olympus, Tokyo, Japan]) and with the DeltaVision RT system, in which images were acquired at 20°C with an automated Olympus IX71 microscope and oil-immersed objectives. Using software Rx. 3.4.3 (Applied Precision, Seattle, WA), images from stacks of >30 *z*-sections of 0.2 μ m were deconvolved with 10 cycles and then projected along the *z*-plane as described previously (Ouasti *et al.*, 2007). For time-lapse videomicroscopy, cells previously labeled with various probes were seeded at $\sim 5 \times 10^6$ /ml in RB onto round coverslips coated with poly-lysine (MatTeK, Ashland, MA). Raw data were acquired after ~ 8 min of FasL addition within a thermostated chamber (37°C), by using a Leica DMIRE2 microscope, a high-sensitivity camera (Photometrics cascade II) and 63 $\times$ or 100 $\times$ objectives. Images were processed with ASMDW Y1.21 software (Leica).

Evaluation of Cell Death

To assess the levels of incipient cell death that occurred under the same conditions as those used for endocytosis studies we used visual inspection of irreversible membrane blebbing (termed “terminal”) as described previously (Stinchcombe *et al.*, 2001). This early hallmark of Fas-induced death (Weis *et al.*, 1995) was evaluated by integrating image analysis of fixed cells with a wealth of live cell images of prolonged experiments in which Fas stimulation led to loss of mitochondrial and cell integrity (cf. Matarrese *et al.*, 2008). The cumulative scoring of terminal blebbing by independent observers correlated well with positive staining for caspase-3 activation.

Other Assays

Activation of caspase-8 was evaluated by using either flow cytometry (Ouasti *et al.*, 2007) or cytofluorescence after loading cells with 10–20 μ M Rho-IETD-bis (Packard *et al.*, 2001). The same substrate was used for measurements with a plate reader (Fluoroskan Ascent; Thermo, Basingstoke, United Kingdom) of caspase activation in cell subfractions (Ouasti *et al.*, 2007). Phagocytosis was evaluated using Congo-red stained yeast (Lugini *et al.*, 2003), whereas macropinocytosis was measured using fluid-phase tracers. The uptake of fixable Ruby-dextran (10 kDa; Sigma Chemie) was undertaken as reported previously (Nichols *et al.*, 2001) and evaluated as described previously (Sabharan-jak *et al.*, 2002), whereas steady-state traffic of transferrin (Tf) was followed with Alexa594-conjugated transferrin (Blanchard *et al.*, 2002; Naslavsky *et al.*, 2004). HPA and wheat germ agglutinin (WGA) conjugates were used as broad markers of membrane traffic, as well as for surface cell decoration (Degli Esposti, 2008).

Image Analysis

Images were processed with the program ImageJ (National Institutes of Health, Bethesda, MD; <http://rsb.info.nih.gov/ij/>) or occasionally with the software routine of the DeltaVision RT program (with colocalization threshold set at 50). Quantitative analysis of colocalization was routinely undertaken using the plugin “colocalization threshold” of ImageJ, which uses the threshold algorithm of Costes *et al.* (2004) (http://www.uhnresearch.ca/facilities/wcif/imagej/appendix_ii). This yielded two complementary but

independent parameters, the single-channel specific Mander's coefficient adjusted for threshold, tM1 or tM2, and Pearson's correlation index of global colocalization (Rtot). Mander's tM1 and tM2 coefficients are normalized to the total pixel intensity (appropriately subtracted for background) of each channel and are thus independent of the absolute intensity of channel fluorescence (Costes *et al.*, 2004). Conversely, Pearson's index represents the *r* of all nonzero-zero pixels that overlay in the images of the channels. It is independent on background but sensitive to individual channel intensity and has values systematically lower than Mander's coefficients, in part because it ranges from a theoretical minimum of -1 to a maximum of 1 (Ménager *et al.*, 2007), whereas Mander's coefficients vary from an unbiased threshold equivalent to a Pearson's value of 0 to a maximum value of 1. RGB.tif files of deconvolved z-projections were split in 8-bit images with 256 channels of grayscale intensity for each color and then cleared of background using a region of interest (ROI) outside cells and the "subtract background from ROI" routine in ImageJ. Complete elimination of bleed-through and nonspecific background was attained with a scaling factor of 10, as verified using costaining of red and green HPA, or red and green secondary antibodies after differential staining of endogenous FasL. The standard settings of threshold colocalization are listed in the legend of Figure 2.

Evaluation of CD59 and CD81 Spreading

We have evaluated the internalization and intracellular distribution of fluorescent conjugates of monoclonal antibodies specific for diverse surface proteins CD59 and CD81. The constitutive traffic of these proteins follows different portals of endocytosis (Fritzsching *et al.*, 2002; Naslavsky *et al.*, 2005; Meertens *et al.*, 2006). Three independent observers undertook morphological analysis of high-resolution images from at least four separate experiments and scored cells to have "spreading" when they exhibited antibodies staining that was strongly altered from the normal surface pattern. The levels of this spreading were highly correlated with colocalization values of the antibodies staining and subunit B of cholera toxin (CtxB).

Statistical Analysis

Results were presented as either histograms containing the mean $\pm$ SE or in interval plots defining the 95% confidence in data variation. Statistical significance of the difference between samples was undertaken with the nonparametric Mann-Whitney test and, whenever normal distribution was evident, also with parametric tests such as analysis of variance (ANOVA), by using the software package MiniTab15 (www.4ulr.com/products/statisticalanalysis). Statistical significance was considered strong when at least two independent tests yielded levels of significance or *p* values <0.05 .

RESULTS

Fas Stimulates Only Some Portals of Endocytosis in T Cell Lines

In our endeavor to understand the mechanism of Fas-enhanced membrane traffic, we first excluded that Fas stimulation could alter the basal level of phagocytosis and macropinocytosis in T cell lines (Supplemental Figure S1, cf. Table 1). We additionally found that Fas stimulation with either its cognate ligand or the agonist antibody CH-11 equally enhanced a wave of endocytosis that was caspase independent, because genetic ablation of caspase-8 or inhibition with the pan-caspase reagent z-VAD did not modify Fas-enhanced internalization of dextrans (Matarrese *et al.*, 2008) and HPA (Degli Esposti, 2008). HPA has been used here as the reference membrane marker because it can access multiple pathways of membrane traffic, similarly to other lectins such as WGA (Vetterlein *et al.*, 2002; Cresawn *et al.*, 2007; Degli Esposti, 2008).

Fas Activation Enhances More Pinocytosis than Clathrin-dependent Endocytosis

Given that previous studies have reported that Fas signaling increases either clathrin-dependent endocytosis (Austin *et al.*, 2006; Lee *et al.*, 2006; Kohlhaas *et al.*, 2007) or pinocytosis (Kenis *et al.*, 2004; Matarrese *et al.*, 2008), we undertook a direct comparison of these different portals in Jurkat cells. Red fluorescent markers were incubated under conditions of steady-state equilibrium with external blue HPA, allowing rigorous colocalization analysis be-

cause of the large separation in the fluorescence of the probes. To label an established portal of clathrin-independent endocytosis, we initially used Ruby-dextran, which had been characterized previously as a fluid-phase marker of endocytic elements internalized via clathrin- and dynamin-independent endocytosis (Sabharanjak *et al.*, 2002; Kirkham *et al.*, 2005; Cheng *et al.*, 2006). Confirming flow cytometry studies (Supplemental Figure S1 and Matarrese *et al.*, 2008), images of Jurkat cells showed a clear increase in the uptake of Ruby-dextran after FasL treatment (Figure 1, A and B). A few vesicles loaded with Ruby-dextran were also positive for endocytosed HPA (Figure 1A, arrow). Although quantitative analysis (Costes *et al.*, 2004) indicated modest levels of overall colocalization between Ruby-dextran and blue HPA in FasL-treated cells, these levels were highly significant because untreated cells exhibited a low uptake of the dextran, with consequent negligible colocalization (Supplemental Figure S2A). Of note, Ruby-dextran is extensively regurgitated early after internalization (Chadda *et al.*, 2007).

We next studied clathrin-dependent endocytosis with fluorescently labeled Tf (Blanchard *et al.*, 2002; Austin *et al.*, 2006). Tf staining displayed a combination of cortical and juxtanuclear distribution, but only the latter increased after 30 min of FasL treatment (Figure 1C). Colocalization of internalized HPA with Tf-labeled elements was limited and concentrated around the Golgi region, in which recycling endosomes cluster (an average Pearson's index of 0.14 was determined in the experiment of Figure 1C, *n* = 14 cells). Consequently, Fas-mediated changes in Tf distribution looked similar to the polarized traffic of Tf receptors that has been observed after T cell activation (Blanchard *et al.*, 2002). By analogy, Fas-induced polarization of Tf distribution (Figure 1C) could reflect enhanced entry into recycling endosomes, in which different portals of endocytosis empty their cargos (Sabharanjak *et al.*, 2002; van Ijzendoorn, 2006). Hence, early Fas signaling increased the uptake of a pinocytic marker that follows clathrin-independent endocytosis (Mayor and Pagano, 2007) more than that of Tf, the classical marker of clathrin-dependent endocytosis. However, Fas also polarized membrane traffic toward the region containing recycling endosomes, thereafter labeled "peri-Golgi" (Supplemental Figure S2B).

Fas Stimulation Induces Peri-Golgi Migration of HPA

To efficiently visualize membrane traffic around the peri-Golgi region of T cells, we subsequently used markers that permanently bind to membrane components like fluorescent derivatives of CtxB, which is rapidly internalized following clathrin-independent routes converging toward recycling endosomes around the Golgi (Sabharanjak *et al.*, 2002; Kirkham *et al.*, 2005; Cheng *et al.*, 2006; Chadda *et al.*, 2007). These routes did not include caveolin-dependent endocytosis, which normally contributes to CtxB traffic (Kirkham *et al.*, 2005; Chadda *et al.*, 2007), because T cells do not express caveolin (Deckert *et al.*, 1996; Orlandi and Fishman, 1998). In Jurkat and primary T cells, CtxB traffic reached equilibrium within 30 min of incubation (Figure 2). At longer times of Fas stimulation, CtxB staining became progressively scattered toward the cell periphery, following the dispersal of Golgi-related membranes that depends upon caspase activation (Ouasti *et al.*, 2007; Degli Esposti, 2008). Hence, the characteristic peri-Golgi distribution of CtxB (verified by counterstaining with the Golgi-specific marker GM130; Supplemental Figure S2B)

provided a valuable internal reference for observing membrane traffic changes before caspases became activated.

Costaining of CtxB with endocytosed HPA indicated a consistent pattern of increased colocalization in Fas-stimulated cells (Figure 2). FasL treatment increased the uptake of CtxB producing an accumulation in the peri-Golgi region that seemed “hypertrophic” with respect to control conditions (Figure 2A). In this region, endocytosed HPA-labeled elements became strongly colocalized with CtxB-labeled membranes (Figure 2A). Using the threshold approach to evaluate pixel colocalization (Costes *et al.*, 2004), the mean

value for threshold-adjusted Mander’s coefficient (tM1) of red HPA over green CtxB increased more than twofold after FasL treatment (Figure 2B, significant at 0.04, Mann–Whitney test). The converse tM2 values for colocalization of green CtxB over red HPA also increased, albeit with weaker statistical significance than tM1 values (Figure 2B). However, complementary quantitative analysis using Pearson’s index of overall colocalization (Rtot), which is distinct and has a wider spread of potential values than Mander’s coefficients, showed a highly significant increase in the overall colocalization of HPA with CtxB after Fas stimulation (Figure 2C).

Figure 1. Endocytosis of dextran is more enhanced than that of transferrin in FasL-treated cells. (A) Jurkat T cells were incubated in RB with 10 μ g/ml Alexa350-HPA (external blue HPA) and 1 mg/ml Ruby-dextran (10 kDa) in the absence or presence of 0.5 μ g/ml FasL for 30 min at 37°C and then washed in the cold before plating and fixing. Images were acquired by DeltaVision RT deconvolution microscopy with a 60 $\times$ oil immersion objective and resulted from projections of 35–40 0.2- μ m z-stacks (Ouasti *et al.*, 2007). (B) The histograms show the numbers of red dextran-positive cells counted as described previously (Sabharanjak *et al.*, 2002) in one of three similar experiments; >80 cells were imaged at low magnification per sample. (C) Jurkat cells were incubated with blue HPA and FasL as described in A; after centrifugation in the cold, they were then resuspended in RB containing 5 μ g/ml Alexa594-conjugated Tf for 10 min at 37°C (including attachment on coverslips) and fixed before imaging, which was acquired by DeltaVision RT deconvolution microscopy under identical settings for all samples. The panels on the far right show colocalization pixels (in white) calculated with the routine of the DeltaVision RT software. The resulting images are equivalent to those obtained with the threshold settings used in Supplemental Figure S2A.

In permeabilized cells, HPA effectively labeled the same peri-Golgi region in which CtxB accumulated (Perez-Vilar *et al.*, 1991; Ouasti *et al.*, 2007). Triple labeling of permeabilized cells indicated that Fas stimulation strongly increased the colocalization of endocytosed red HPA with both green CtxB and internal blue HPA (Figure 3, A and B). This occurred without altering the strong colocalization of CtxB with internal HPA, because their average threshold-adjusted Mander's coefficient varied from 0.684–0.657 after FasL treatment. The increased peri-Golgi distribution of internalized HPA was additionally confirmed in dual HPA experiments (cf. Ouasti *et al.*, 2007). The same experiments conducted in caspase-8–deficient Jurkat cells showed equivalent results of strong colocalization between internalized HPA and internal HPA (Figure 3C, right histograms), confirming that

Fas-enhanced colocalization of endocytic and Golgi-related membranes was independent of apical caspases.

To further verify the polarization of endocytosed HPA toward the peri-Golgi region, we next undertook live cell imaging of Jurkat cells attached to polylysine-coated wells after costaining with red HPA and green CtxB. Although wide-field images obtained in this way lacked the resolution of deconvolved images of fixed cells, they provided clear evidence for dynamic movements of HPA-labeled membranes toward CtxB-labeled membranes after treatment with FasL (Figure 3D).

Fas Stimulates the Traffic of CD59

We next followed the cellular distribution of fluorescently labeled antibodies specific for CD59, an abundant protein

Figure 2. Fas alters the traffic of cholera toxin B. (A) Live Jurkat cells were treated with FasL for 30 min in the presence of 2 $\mu\text{g}/\text{ml}$ tetramethylrhodamine B isothiocyanate-conjugated HPA (red) together with 1 $\mu\text{g}/\text{ml}$ Alexa488-conjugated CtxB (green). Deconvolved images were acquired with a 100 $\times$ objective under identical settings for all samples and were analyzed with the threshold colocalization plugin (see *Materials and Methods*), excluding constant intensity for colocalized pixels and zero-zero pixels in the calculations. The plugin generated the RGB images shown, in which the relative intensity of colocalized pixels was rendered in 255-level grayscale merged with the red and green channel. Bars, 5 μm . (B) Colocalization analysis was undertaken using the same settings as described in A; data represent mean values $\pm$ SE from four separate experiments. The Mann–Whitney test indicated a difference significant at 0.007 for tM1 (red HPA over green CtxB) and 0.06 for tM2 (green CtxB vs. red HPA), but the latter also showed $p < 0.05$ with two-sample t test and ANOVA tests. (C) Different cell images ($n = 15$) were analyzed in an experiment equivalent to that described in A, in which Fas was stimulated by the agonist antibody CH-11 for 30 min. The plot represents 95% confidence intervals of the Pearson's index of overall colocalization, R_{tot} , which is independent of computed threshold values (Costes *et al.*, 2004); statistical tests indicated a 0.0013 level of significance with Mann–Whitney and $p = 0.001$ with two-sample t test.

of T cells (Deckert *et al.*, 1996) that is anchored to the exterior of the plasma membrane via glycosyl-phosphatidylinositol (GPI). As for other GPI-anchored proteins, the

constitutive traffic of CD59 is specifically driven by clathrin- and dynamin-independent portals of endocytosis (Nichols *et al.*, 2001; Sabharanjak *et al.*, 2002; Naslavsky *et al.*,

Figure 3. Intracellular changes of HPA versus CtxB. (A) Samples were treated as in the experiment of Figure 2A, but after fixation they were quenched with unlabeled HPA (to minimize surface staining), permeabilized with Triton X-100, and stained with 2 μ g/ml blue HPA to label Golgi-related membranes (Degli Esposti, 2008). Deconvolved images were acquired with a 100 $\times$ objective. Bar, 5 μ m. (B) Detailed evaluation of colocalization levels (mean $\pm$ SE) was undertaken as in Figure 2B by using three or more cell images as those described in A; asterisks note high levels of statistical significance (<0.02 , Mann–Whitney test). (C) Values of colocalization were obtained in separate experiments of dual HPA staining (cf. Ouasti *et al.*, 2007) by using both wild-type (histograms on the left) and caspase-8-deficient Jurkat cells (on the right). A 0.03 level of significance was computed for the difference between control and FasL-treated cells (Mann–Whitney test). (D) Single frames of time-lapse video images (63 $\times$ objective) of cells costained with red HPA and green CtxB as in the experiment of Figure 2A, treated with FasL, and then acquired after focusing on a selected field area, the initial bright-field image of which is shown on the left.

Figure 4. Fas stimulation alters CD59 traffic. (A) Low-magnification images (nondeconvolved 60 $\times$, to show the overall appearance of cell staining) were acquired for cells labeled with a nonactivating anti-CD59-FITC (1:10) and red HPA before (control) and after 30-min incubation with FasL. Arrows indicate peri-Golgi accumulation of staining. The corresponding differential interference contrast image is shown on the right. (B) Deconvolved images were obtained with a 100 $\times$ objective under the same conditions as in the experiment in A. Arrows point to colocalized membrane elements around the peri-Golgi region. (C) Interval plot of Pearson's index of colocalization for $n = 9$ (control) and $n = 20$ (+FasL) cells from the experiment in B. The distribution of values was significantly different at 0.0014 (Mann-Whitney test). (D) Typical staining of Jurkat cells with FITC-CD81 antibody equilibrated 30 min before and after the addition of FasL for 30 min as described in B. Note the limited colocalization with internalized red HPA. Bars, 5 μ m (B and D).

Figure 5. Fas-induced changes in the traffic of CD59 relative to that of HPA and CtxB. (A) Images were obtained as in the experiment of Figure 4A. The panels on the far right show the profile of the colocalized pixels (gray) obtained with the threshold plugin applied to the merged channels of endocytosed HPA (red) and internalized CD59 (green). (B) Panels show representative images obtained under the same conditions as described in A except that PE/Cy5-conjugated anti-CD59 (red) was costained with green CtxB as in the experiment of Figure 2. (C) Histograms represent the mean values $\pm$ SE from three separate experiments such as that in A (green histograms, reporting threshold-adjusted Mander's coefficient of CD59 green over red HPA) and that in B (red histograms, reporting threshold-adjusted Mander's coefficients of CD59 red over green CtxB). Values of FasL-treated cells were significantly different at 0.0265 (tM2) and 0.0074 (tM1) by using the Mann-Whitney test. Bars, 5 μ m. (D) Time-lapse images (obtained with a 100 $\times$ objective as in the experiment of Figure 3D) were selected from Supplemental Movie 1. Cells were costained with red HPA and green CtxB as in Figure 4A. Note the ejection of a costained membrane particle (red arrow) after intracellular convergence of staining (white arrows). Bars, 5 μ m (A and B).

Figure 6. Fas-enhanced traffic of CD59 is blocked by Rho GTPase inhibitors. (A) The plots represent 95% confidence intervals in the colocalization between CD59 and CtxB in $n > 6$ cell images from an experiment equivalent to that of Figure 5B. Control cells were treated for 30 min with a nonactivating Fas antibody, whereas other cells were treated for 30 min with CH-11 in the absence (CH-11 alone) or presence of 50 μ M z-VAD, 20 μ M secramine A, or 100 ng/ml *C. difficile* toxin B (all these compounds were preincubated for 30 min before Fas activation). Control cells with inhibitors showed values within the interval of untreated cells. The difference with respect to the control sample was significant at 0.008 for CH-11 alone and 0.002 for CH-11 + z-VAD (Mann-Whitney test) but not for the other samples (e.g., 0.83 with CH-11 plus secramine). Conversely, the difference with respect to CH-11 alone was significant at 0.024 and 0.017 for the sample plus secramine and that plus *C. difficile* toxin B, respectively. Of note, in a parallel flow cytometry

experiment secramine reduced the uptake of CtxB by 30%. (B) Pharmacological profile of cell spreading of CD59 was evaluated by combining the independent scoring of three observers. The cellular distribution of anti-CD59 fluorescent antibodies was undertaken in high-resolution images and cells exhibiting CD59 staining that was strongly altered from the normal surface pattern were scored to present "spreading" (for increased intracellular distribution). Histograms represent the average $\pm$ SE of the indicated number of experiments. Differences were statistically significant at <0.03 (Mann-Whitney test). (C) Mean values of cell spreading of CD81 (cf. Figure 4D) were evaluated in $n = 4$ experiments by using the same morphological scoring as that for CD59 and represented with the same scale as described in B. Fas stimulation did not significantly change the predominantly superficial staining of CD81 ($p > 0.3$ with either Mann-Whitney or ANOVA tests).

2004; Mayor and Pagano, 2007). Moreover, CD59 distribution is normally restricted to clusters lying at the surface of Jurkat cells (Deckert *et al.*, 1996). Hence, we surmised that CD59 could be an ideal reporter for evaluating Fas-induced changes in constitutive endocytosis.

The surface-confined distribution of fluorescent anti-CD59 antibodies was lost soon after FasL treatment due to rapid internalization, which was followed by extensive redistribution in the cell interior (Figure 4, A and B). Within 30 min of Fas stimulation, several cells presented with CD59-positive membranes converging onto endocytosed HPA, increasing their colocalization around the peri-Golgi region (Figure 4, B and C). As a pertinent control, we followed the internalization of fluorescent antibodies specific for CD81, a surface protein trafficking predominantly via clathrin-dependent endocytosis (Fritzsche *et al.*, 2002; Meertens *et al.*, 2006). Contrary to CD59, CD81 remained confined in cortical and

surface elements after Fas-stimulation, showing very little internal colocalization with HPA (Figure 4D). The specific accumulation of CD59 in the peri-Golgi region was subsequently confirmed, also quantitatively, in costaining experiments with CtxB (Figure 5).

Fas-induced alterations included an increased presence of anti-CD59 antibodies in external protrusions, some of which were shed outside the cell body (Figures 4A and 5D and Supplemental movie 1). Plasma membrane shedding (sometimes called "ectocytosis") has been described previously in apoptotic cells (Distler *et al.*, 2005; Pilzer *et al.*, 2005) and is reported to be present in Fas-stimulated Jurkat cells too (Elward *et al.*, 2005). However, the release of membrane particles enriched in CD59 was strongly dependent upon the activation of apical caspases (Elward *et al.*, 2005). Blocking caspases by the general inhibitor z-VAD not only reduced Fas-induced ejection of mem-

Figure 7. Rho-GTPase inhibition abolished Fas-induced changes in CD59 distribution. (A) FasL treatment (15 min) increased the endocytic traffic of green CD59 coincubated with red HPA as in Figure 4. FasL did not change the cellular distribution of CD59 after pretreatment with 0.1 $\mu\text{g}/\text{ml}$ *C. difficile* toxin B (bottom). Deconvolved images were taken with a 100 $\times$ objective. (B) Representative 60 $\times$ images of internalized CD59-FITC staining before (top) and after (bottom) 30-min incubation with FasL in the absence or presence of 20 μM secramine, incubated 30 min before Fas stimulation. Bars, 5 μm .

Figure 8. Fas enhances the convergence of active caspases in the peri-Golgi region. (A) Treatment with superFasL was undertaken in cells colabeled with green CtxB and blue HPA as in the experiments of Figures 1 and 2. After 50-min incubation, Jurkat cells were attached to

brane particles but also enhanced the internalization of anti-CD59 antibodies (Figure 6A and Supplemental Figure S3A). Importantly, Fas stimulation enhanced CD59 internalization also in primary activated T lymphocytes (Supplemental Figure S3B). This indicated that rapid alteration of CD59 traffic was a general response of Fas-stimulated T cells.

Fas Stimulates Rho GTPase-dependent Endocytosis

We next studied the mechanism of Fas-induced alteration of CD59 distribution. Clathrin-independent endocytosis trafficking CD59 requires actin-modulating Rho GTPases (Sabharanjak *et al.*, 2002; Kirkham *et al.*, 2005; Cheng *et al.*, 2006), which are cumulatively blocked by toxin B of *C. difficile* (Mayor and Pagano, 2007). Pretreatment with *C. difficile* toxin B at concentrations that blocked Rho GTPases without impairing viability (Audoly *et al.*, 2005) strongly inhibited Fas-induced internalization of CD59 and its colocalization with CtxB and HPA (Figures 6 and 7). Complementary results were obtained by quantitative analysis of colocalization between CD59 and CtxB (Figure 6A) and by the scoring of cellular spreading of CD59-labeled membranes that, contrary to that of CD81, strongly increased in Fas-stimulated cells (Figure 6, B and C). Indeed, CD59 staining hardly changed before and after Fas stimulation when the toxin was present, similarly to the unaltered pattern of CD81 staining (Figure 7A and Supplemental Figure S4A). We verified that *C. difficile* toxin predominantly blocked CDC42 by using secramine A, a compound that specifically inhibits CDC42 (Pelish *et al.*, 2006). Results with secramine A were essentially superimposable to those obtained with *C. difficile* toxin B, following the colocalization between CD59 and CtxB (Figure 6A) and the internal distribution of CD59 (Figures 6B and 7B).

Colocalization of Fas and Caspases with HPA in the Peri-Golgi Region

The results obtained so far underlined a common feature in Fas-induced alterations of membrane traffic, namely, its centripetal convergence toward the peri-Golgi region. We next

addressed the question as to whether this related to documented differences in the traffic of internalized Fas receptors between Jurkat and type I cells (Algeciras-Shimmich *et al.*, 2002; Eramo *et al.*, 2004; Söderström *et al.*, 2005; Lee *et al.*, 2006). In type I cells, ligated death receptors and their associated DISC follow rapid clathrin-dependent internalization, ending up into late endosomes/lysosomes. This was marginally present in Jurkat cells (Lee *et al.*, 2006), whereas other type II cells showed limited or delayed internalization of death receptors (Eramo *et al.*, 2004; Austin *et al.*, 2006; Matarrese *et al.*, 2008).

To follow the path of ligated CD95/Fas in T cells, we first analyzed the internalization of FasL added at concentrations saturating its cognate receptor (Figure 8A, cf. Eramo *et al.*, 2004). After 40–60 min, a proportion of FasL bound to Fas was found in intracellular elements that colocalized with either CtxB (Figure 8A) or endocytosed HPA (Figure 8, A and B; also see Figure 9A and Supplemental Figure S4B). Because DISC assembly and recruitment of procaspase-8 occur also during receptor internalization, the intracellular location of ligated Fas coincides with the initial intracellular activation of apical caspases (Lee *et al.*, 2006). We then studied the subcellular distribution of active caspases using a fluorogenic substrate nominally specific for caspase-8, Rhodamine110-IETD-bisamide (Rho-IETD-bis), enabling direct measurements of caspase activation within cells (Packard *et al.*, 2001). When, occasionally, a control cell displayed bright green staining after loading with Rho-IETD-bis, it also exhibited signs of advanced death with accumulated vacuoles, in part because the substrate reacted with cathepsin D under acidic conditions (S. Ivanova, unpublished data). Besides this background, punctuate staining of Rho-IETD-bis occurred between 30 and 60 min of Fas stimulation, progressively accruing around the peri-Golgi region. Consequently, active caspases became extensively colocalized with endocytosed HPA, as quantitatively verified with the usual colocalization analysis (Figure 8, C and D).

Fas colocalization with HPA was also detected by using direct staining of all the complement of CD95/Fas present within cells and significantly increased in intracellular elements after 40 min of receptor stimulation (Figure 9A). At this time, we consistently detected activation of intracellular caspases with both fluorogenic substrates and immunostaining of the active form of the proteases (Ouasti *et al.*, 2007). By using the latter approach, we followed the intracellular distribution of activated caspase-3, the major substrate of caspase-8 and dominant executioner caspase (Figure 9B). Along the progression of Fas signaling, caspase-3-positive elements moved from the periphery to the interior of cells, accumulating in the peri-Golgi region where they colocalized with endocytosed HPA (Figure 9B). Subsequent to caspase-dependent fragmentation of the Golgi (Ouasti *et al.*, 2007), caspase-3- and HPA-positive membranes continued to be closely associated throughout the cell (Figure 9B, bottom).

Intriguingly, Rho GTPase inhibition marginally reduced the overall intensity of caspase activation within Fas-stimulated cells (Supplemental Figure S4C), indicating that Rho GTPase-dependent routes of membrane traffic were unlikely to contribute to DISC assembly. Did then Rho GTPases influence downstream steps of death propagation? To answer this crucial question, we resorted to analytically determine the levels of terminal blebbing, an early hallmark of Fas-induced death (Weis *et al.*, 1995) that could be evaluated under the same conditions of our endocytosis studies (see *Materials and Methods* and Supplemental Figure S4D). Concomitantly with the initial increase in caspase activation, the

Figure 8 (cont). coverslips, fixed with cold methanol (Siegel *et al.*, 2004), and then treated with a monoclonal for FasL, followed by a Rhodamine X-conjugated secondary to detect ligated CD95/Fas (cf. Eramo *et al.*, 2004). Representative 100× images of untreated (top) and FasL-treated (bottom) cells were obtained after deconvolution. The central panels show the images of colocalized pixels between the red (anti-FasL) and green (CtxB) channels, whereas the right-most panels show the merged RGB images. (B) The two panels were generated by the threshold colocalization plugin and show the colocalized pixels between the red (anti-FasL) and blue (endocytosed HPA) channel of the same images described in A. Quantitative analysis showed an average Pearson's index of ~0 for untreated and 0.162 for FasL-treated cells, respectively (n = 4). (C) Jurkat cells were treated with FasL for up to 1 h in the presence of 10 μM Rho-IETD-bis, producing staining which was predominantly due to the activation of caspase-8. Quantitative evaluation of colocalization for red HPA with Rho-IETD-bis staining of active caspase(s) was undertaken with seven (control) to 10 (FasL-treated for 45 min) cell images, which were analyzed with the threshold method as described in Figure 2. The mean values of threshold adjusted Mander's coefficient were significantly different at 0.0008 (Mann-Whitney test). (D) To evaluate the internalization of active DISC (Lee *et al.*, 2006), we followed the progression of apical caspase activity with the fluorogenic Rho-IETD-bis. The functional staining of caspase-8 progressively polarized toward the peri-Golgi region, as indicated by the images of colocalized pixel in grayscale obtained as described in Figure 2 (panels of the far right). Bars, 5 μm.

Figure 9.

basal level of terminal blebbing increased over 10-fold (Figure 9C). As expected, blocking caspases with z-VAD reduced this indicator of incipient death (Figure 9C). However, also secramine and *C. difficile* toxin B significantly reduced Fas-enhanced terminal blebbing (Figure 9C). Our results thus suggested that Rho GTPases activated early after DISC assembly may contribute to death propagation in T cells, consistent with previous evidence for a link between receptor-stimulated endocytosis and apoptosis signaling (Lee *et al.*, 2006; Matarrese *et al.*, 2008).

DISCUSSION

Although confirmed in multiple reports (Kawasaki *et al.*, 2000; Kenis *et al.*, 2004; Lee *et al.*, 2006; Ouasti *et al.*, 2007; Matarrese *et al.*, 2008), the mechanism and significance of Fas-enhanced endocytosis have remained obscure (Siegel *et al.*, 2004; Austin *et al.*, 2006; Chaigne-Delalande *et al.*, 2008; Reinehr and Häussinger, 2008). Here, we clarify that Fas-stimulated T cells open Rho GTPase-dependent portals that drive the traffic of CD59 (Figures 4–7). Fas signaling also induces a global polarization of endocytic membranes toward the Golgi apparatus, a novel finding with the important implications discussed below.

We have extensively used HPA as a general membrane marker with access to different portals of endocytosis as well as secretory organelles. This marker has been essential for visualizing the peculiar alteration in membrane traffic that Fas stimulation induces in T cells, namely, the accumulation of early and recycling endosomes in the peri-Golgi region. Previously, we reported that FasL-treated cells showed an increased merging of internalized HPA with GM130 and ERGIC-53, membrane markers of the early secretory system (Ouasti *et al.*, 2007). Concomitantly, HPA-labeled membranes also colocalized with mitochondria (Ouasti *et al.*, 2007), which in turn became associated with early endo-

somes (Kawasaki *et al.*, 2000; Matarrese *et al.*, 2008). Other studies have shown an intracellular colocalization of Fas with CtxB (Siegel *et al.*, 2004; Legembre *et al.*, 2005), which typically concentrates in the peri-Golgi region (Figure 2 and Supplemental Figure S2B; cf. Sabharanjak *et al.*, 2002). We can now rationalize all this evidence as an expression of Fas-enhanced endocytic traffic concentrating membrane elements in the peri-Golgi region, in which Golgi membranes and mitochondria cluster together (for review, see Degli Esposti, 2008). Consequently, the intermixing of mitochondria and other organelles observed previously may represent a reporter for the Fas-induced polarization of membrane traffic.

These considerations could be extended to other type II cells but not to type I cells. Abundant evidence obtained after surface down-modulation of CD95/Fas has indicated that type I cells respond to Fas stimulation by enhancing clathrin-dependent endocytosis, along which ligated receptors are rapidly internalized and form a mobile signaling platform (Algeciras-Schimmich *et al.*, 2002; Lee *et al.*, 2006). This pathway of endocytosis ultimately sorts Fas and its associated DISC for endolysosomal degradation, thereby leading to signal attenuation.

Type II cells do not show an equivalent sorting for rapid degradation (Lee *et al.*, 2006; Ouasti *et al.*, 2007), suggesting a diversion of membrane traffic from late endosomes/lysosomes. Confirming this possibility, we demonstrate here that Fas stimulation concentrates and polarizes membrane traffic into the peri-Golgi region of T cells, in which recycling endosomes connect the endocytic pathway to the exocytic pathway (van Ijzendoorn, 2006; Ménager *et al.*, 2007). The traffic diversion into recycling endosomes could provide spatial segregation of Fas signaling into the cell, creating two interconnections: 1) between endocytic elements and exocytosis, which also drives a Fas-induced delivery of new death receptor molecules to the cell surface (Rheiner and Häussinger, 2008); and 2) between endocytic elements and mitochondria, the intermixing of which may facilitate the priming of mitochondrial membranes to the proapoptotic action of Bcl-2 proteins (Matarrese *et al.*, 2008).

Both interconnections underline characteristic features of type II cells, such as the delayed down-modulation of ligated Fas receptors (Eramo *et al.*, 2004; Chaigne-Delalande *et al.*, 2008) and the crucial engagement of mitochondria for caspase amplification (Peter and Krammer, 2003). Moreover, enhanced traffic into recycling endosomes could be instrumental for routing active caspases to selected cellular compartments, in particular secretory organelles where their action promotes outward movement of endomembranes (Elward *et al.*, 2005; Ouasti *et al.*, 2007; Rheiner and Häussinger, 2008). These endomembranes contain newly synthesized Fas that, once delivered to the cell surface, could produce signal persistence by binding to additional FasL molecules. This process would partially compensate for ongoing internalization of ligated receptors and perhaps potentiate intracellular signaling until mitochondria are fully engaged.

In the new view of traffic diversion to recycling endosomes, how would Fas signaling produce a different sorting of endocytic membranes in different cell types? Our simplest explanation is that the assembled DISC promotes activation of Rho GTPases in T and other type II cells but not in type I cells. Besides our data of Rho-GTPase inhibition (Figures 6 and 7), the studies of Subauste *et al.* (2000) and Söderström *et al.* (2005) provide supportive evidence for early activation of CDC42 and related GTPases, which is not present in type

Figure 9 (cont). Fas stimulation enhances its internalization with diffusion of caspase activation and cell death. To evaluate the whole complement of CD95/Fas, we stained cells fixed as in the experiment of Figure 8A by using the FITC-conjugate of the anti-CD95 monoclonal antibody DX2 (Lee *et al.*, 2006). After 40 min of Fas stimulation, the time at which overt caspase-3 activation was detected (Supplemental Figure S4C), an increased proportion of this staining colocalized with internal HPA, as indicated by the images of colocalized pixels obtained with the threshold plugin (panels on the far right). (B) Staining of active caspase-3 was obtained in a parallel experiment with HPA-labeled cells permeabilized with 0.1% saponin, by using a rabbit antibody specific for the active form of caspase-3 followed by an Alexa488-conjugated secondary. The top panels show a cell displaying the typical early distribution of caspase-3-labeled elements at the cell periphery, progressively polarizing toward a compact peri-Golgi region. The bottom panel shows a pattern typical of cells with advanced caspase activation, in which the Golgi region had dispersed and fragmented. Note the sharper green staining than in A, due to the brighter fluorescence of Alexa488 with respect to that of FITC, also enabling more sensitive detection than in previous works (e.g., Eramo *et al.*, 2004). Bars, 5 μ m. (C) The histograms represent the mean $\pm$ SE values of terminal blebbing evaluated in different experiments, scoring an overall set of $\sim$ 60 fields containing >1000 cells per sample. With respect to the sample of cells treated with CH-11 alone, reduction of blebbing was significant at 0.014, 0.037, and 0.008 for + z-VAD, + secramine, and + *C. difficile* toxin B, respectively (using Mann-Whitney test adjusted for ties). The sample + secramine showed a significant difference also using parametric tests ($p = 0.024$ with one-way ANOVA and $p = 0.033$ with two-sample t test; $n = 6$ experiments).

I cells. Moreover, Rho GTPases also reside in recycling endosomes, in which they may further stimulate membrane traffic.

The small Rho GTPase CDC42 predominantly drives Fas-stimulated membrane traffic in T cells, for the following reasons: 1) it promotes the selective traffic of GPI-anchored proteins such as CD59 (Sabharanjak *et al.*, 2002; Mayor and Pagano, 2007), which we found to be rapidly altered after Fas activation (Figures 4–7); 2) it is activated early after Fas stimulation (Subauste *et al.*, 2000), as confirmed in our experiments (unpublished data); 3) it is selectively inhibited by the semisynthetic compound secramine A (Pelish *et al.*, 2006), which abolishes the alteration of CD59 traffic (Figures 6 and 7); 4) it is upstream of Rac in promoting filopodia and other surface protrusions, which are blocked by *C. difficile* toxin B (Malorni *et al.*, 2003), together with the reduction in CD59 traffic alterations (Figure 6); 5) it is the central regulator of cell polarity (Etienne-Manneville, 2004), and we found that Fas stimulation increases the polarization of membrane traffic toward the Golgi region (Figures 2–7); and 6) when expressed in its constitutively active form, it promotes cell death in Jurkat cells (Chuang *et al.*, 1997), and we found that its inhibition with secramine reduces incipient cell death (Figure 9C).

In clarifying the link between endocytosis and the intracellular signaling of Fas, our work opens new perspectives to appreciate the biological role of membrane traffic in the death program of T cells. Of note, polarized recycling of membrane traffic is a key property of activated T cells, which enables intercellular communication with other cells of the immune system (Krummel and Macara, 2006). We are currently investigating the connections between intracellular membrane traffic and intercellular forms of communication during Fas-induced cell death.

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