CD95 capping is ROCK-dependent and dispensable for apoptosis

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Summary

Upon engagement, the CD95 receptor is rapidly clustered into cellular 'caps'. This receptor capping is one of the first events to take place following activation and it has been proposed to be important for the initiation of apoptotic signaling. As the biological roles of CD95 capping are still elusive, we explored in detail the role of capping in induction of apoptosis in lymphocytes. CD95 capping was shown to be uncoupled from apoptosis, as apoptosis could occur in the absence of CD95 capping and, vice versa, capping could occur without inducing apoptosis. CD95 capping occurred concomitantly with reorganization of the actin cytoskeleton and aggregation of lipid rafts. While inhibition of actin polymerization and caspase-8 activity had cell type-specific effects on capping in type I and type II cells, the rapid CD95-mediated cellular polarization, as visualized by the orchestrated reorganization of CD95, F-actin and lipid rafts, was shown to be dependent on signaling by Rho kinase (ROCK) in both cell types, however, by distinct activation mechanisms in the respective cell type. CD95 activated RhoA exclusively in the type II cell, whereas ROCK activation was caspase-dependent in the type I cell. Taken together, our results imply that CD95 capping and the subsequent cellular polarization is a ROCK signaling-regulated process that does not correlate with the induction of apoptosis, but is more likely to be involved in the emerging non-apoptotic functions of CD95.

Key words: CD95, Receptor capping, Apoptosis, Rho kinase, RhoA, Actin, Cytoskeleton, Lipid raft, T cells

Introduction

CD95 (Fas/Apo-1) (Itoh et al., 1991) is the most common death receptor in the TNF receptor family. It is a type II transmembrane protein broadly expressed in a variety of cells and tissues, and it has the capacity to mediate rapid apoptosis when triggered in cells that are receptive to its apoptotic signaling. CD95 is especially important in the immune system, where it regulates the persistence and resolution of immune responses. Upon triggering of CD95, the Fas-associated death domain protein (FADD) and caspase-8 are rapidly recruited to the intracellular part of the receptor, thereby forming the death-inducing signaling complex [DISC; Kischkel et al. (Kischkel et al., 1995)]. In the DISC, caspase-8 is rapidly activated and can then cleave cytoplasmic substrates with high specificity. Two pathways for CD95 signaling have been described and are referred to as type I and type II (Scaffidi et al., 1998; Scaffidi et al., 1999). In type I cells, a high quantity of DISC is rapidly formed upon stimulation and efficient apoptosis is thereby induced. In type II cells, formation of the DISC is a limiting factor for the apoptotic signal, which is therefore amplified through a signaling loop that involves the pro-apoptotic Bcl-family protein Bid and the mitochondria. While these two modes of apoptotic signaling from death receptors are fairly well established, the molecular mechanisms involved in these two signaling pathways are not understood in detail.

In addition to the ability of CD95 to induce apoptosis, non-apoptotic functions for CD95 are now emerging. CD95 has recently been shown to induce T cell co-activation, proliferation (for a review, see Wajant et al., 2003), differentiation (Rescigno et al., 2000), inflammation (Miwa et al., 1998; Park et al., 2003), migration (Ottonello et al., 1999; Seino et al., 1998), neurite outgrowth (Desbarats et al., 2003), integrin expression (Jarad et al., 2002), hepatic regeneration (Desbarats and Newell, 2000) and inflammatory angiogenesis (Biancone et al., 1997). Furthermore, both FADD and caspase-8 have been shown to be required for the activation and proliferation of T cells, which in some instances can even be delivered by CD95 (for a review, see Newton and Strasser, 2003). The involvement of these apoptotic mediators in both proliferative signaling and apoptosis makes CD95 a highly interesting receptor and implies that tight regulatory mechanisms must be in place to direct the cell to either survival or apoptosis.

The mechanisms regulating the balance between death receptor-mediated apoptotic and non-apoptotic signaling are not clearly understood. The Caspase-8 inhibitory protein FLIP (Hu et al., 2000; Kataoka et al., 2000), MAPK/ERK signaling (Holmström et al., 1999; Ahn et al., 2001; Tran et al., 2001), and NF-κB activation (Ponton et al., 1996) have all been implicated in non-apoptotic signaling by CD95.

Capping of immune cell receptors is important for a broad range of cellular functions, such as T cell activation,

costimulation and migration. Receptor triggering is then often followed by capping, reorganization of the cytoskeleton and cellular polarization (for reviews, see Sanchez-Madrid and del Pozo, 1999; Serrador et al., 1999). These generalizations also apply to CD95, as one of the first events to occur upon ligation of CD95 in lymphocytes is capping of the receptors. CD95-mediated signaling is reflected by and also affected by dynamic changes in the cytoskeleton. Indeed, it has been suggested that CD95 is indirectly bound to actin via the cytoskeletal linker protein ezrin and that the organization of the microfilaments affects the outcome of CD95 stimulation (Parlato et al., 2000). CD95 responses are also regulated by intermediate filaments, as the keratins 8 and 18 provide protection against CD95-mediated apoptosis (Gilbert et al., 2001). It is, therefore, evident that the cytoskeleton is tightly linked to CD95 signaling. In this sense, it seems more than plausible that the cytoskeleton could regulate the lateral mobility and distribution of CD95, and thereby the extent of capping, which in turn could affect the outcome of the signal triggered by the receptor. However, the exact mechanisms by which CD95 and the cytoskeleton cooperate are poorly understood and require further investigation.

CD95 capping and signaling have been shown to involve membrane lipid rafts. Lipid rafts act as platforms on the cell surface integrating signaling pathways (Simons and Ikonen, 1997). It is not clear whether CD95 is constantly associated with lipid rafts or if CD95 translocates to the rafts upon receptor stimulation. Assembly of the CD95 DISC has been proposed to occur within the lipid raft (Scheel-Toellner et al., 2002; Garofalo et al., 2003; Grassme et al., 2003). CD95 translocation into lipid rafts is also associated with ligand-independent triggering of CD95 (Gajate and Mollinedo, 2001). Aggregation of lipid rafts has been shown to be important for many functions, especially in immune cell signaling, such as targeted transportation of membrane proteins (Millan et al., 2002) and formation of immune synapses (Jordan and Rodgers, 2003). Membrane lipid rafts have also been shown to be present in the leading edge during cell migration and to mediate front-rear polarity in cells (Manes et al., 1999). There seems to be a tight relationship between actin reorganization, aggregation of lipid rafts and receptor signaling during both migration and T cell activation (Valensin et al., 2002). However, the knowledge about the functions of lipid rafts and their aggregation in modulating CD95 responses remains poor.

The role of CD95 capping is still unclear. While it has been suggested to be ceramide-dependent and essential for CD95-mediated apoptosis (Cremesti et al., 2001; Grassme et al., 2001a), there are also reports of CD95-mediated ceramide generation in the absence of apoptosis (Grullich et al., 2000; Hsu et al., 1998) and CD95-mediated apoptosis without generation of ceramide (Watts et al., 1997). There are also indications that pro-apoptotic capping could be important for CD95 internalization (Algeciras-Schimnich et al., 2002). In contrast, there are recent results suggesting that constitutive CD95 capping could be involved in mediating survival rather than apoptosis, as sequestering of CD95 and the associated caspase-8 into clusters would be a way to secure the receptor from initiating an apoptotic signal (Strauss et al., 2003).

As the role of, and the mechanisms underlying, CD95 capping were unclear, we wanted to study how capping occurs and what the relationship is to induction of apoptosis. As a hypothetical regulator for CD95 organization, which is known to regulate both cytoskeletal structure (for a review, see Narumiya et al., 1997) and immune cell receptor capping (Tharaux et al., 2003), we picked the serine/threonine kinase Rho kinase (ROCK). ROCK signaling has been implicated in multiple cellular functions, such as T cell activation, proliferation (Tharaux et al., 2003), migration (Honing et al., 2004), adhesion and apoptosis (for a review, see Riento and Ridley, 2003), all functions that can also be mediated by CD95 signaling. In this sense, ROCK is a plausible regulator of both CD95 organization and signaling.

We observed that CD95 capping occurs concomitantly with polarization of F-actin and lipid rafts. We also found that CD95 capping is not essential for the induction of apoptosis and that capping can occur even when apoptosis is inhibited. CD95 capping, but not the induction of apoptosis, was shown to be dependent on ROCK signaling. Since we found that CD95 capping is not required for induction of apoptosis, our results indicate that capping could regulate other functions of CD95 that require ROCK-dependent cellular polarization.

Materials and Methods

Cell culture

The human leukemic T cell lines, Jurkat (clone E6-1) and H9 were obtained from ATCC (Maryland, USA). The cells were cultured in RPMI 1640 medium supplemented with 10% (Jurkat) or 20% (H9) fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in 5% CO_2 in air. The cells were kept at a density of 0.5-1.0×10^6/ml.

Jurkat or H9 cells were incubated at a density of 1×10^6/ml with or without 200 ng/ml agonistic anti-human CD95 IgM antibody (CH11; MBL, Watertown, MA, USA) for the indicated time periods in the absence or presence of 20 μM cytochalasin D (cytD; Calbiochem, San Diego, CA, USA), 20 μM zIETD-fmk (Sigma), or 20 μM Y-27632 (Calbiochem).

Analysis of CD95, F-actin and lipid rafts

1.5×10^6 untreated or pre-treated cells were incubated with 0.5 μg/ml of αCD95 (CH11) on ice for 30 minutes, washed twice with RPMI-FCS and incubated for 30 minutes with Alexa Fluor 488 anti-mouse antibody (1:400; Molecular Probes, Eugene, OR, USA). After washing in ice-cold RPMI-FCS, the labeled cells were transferred to a 37°C water bath for the indicated time periods. The stimulation was stopped by transferring samples onto ice. Cells where then washed once in ice-cold PBS and fixed for 15 minutes in cold 3% paraformaldehyde. Cytospin (Thermo Shandon, USA) preparations were finally made and mounted in Vectashield (Vector Laboratories, Burlingame, CA, USA).

For colocalization studies of CD95 with F-actin or lipid rafts, cells were labeled for CD95 as described above and the fixed cytospin preparations were then permeabilized with 0.1% Triton X-100 for 10 minutes and blocked in 0.5% BSA/PBS for 30 minutes. Then the cells were labeled with Alexa Fluor 546 phalloidin (Molecular Probes, Eugene, OR, USA) or Alexa Fluor 555-conjugated cholera toxin subunit β (CTxB; Molecular Probes) for 30 minutes and then washed three times with PBS and mounted in Vectashield (Vector Laboratories).

Confocal microscopy

Images were collected using a Zeiss LSM 510 META laser scanning confocal microscope (Zeiss, Jena, Germany) configured on an inverted Axiovert 200M stand (Zeiss) equipped with a Plan-Apochromat 63×/1.4 oil DIC objective. Alexa Fluor 488 fluorescence

was excited at 488 nm with an argon ion laser and emission was recorded through a 500-530 nm IR band-pass filter. Alexa Fluor 555 or 546 fluorescence was excited at 543 nm with a helium-neon laser and emitted light was recorded through a 560 nm long-pass filter. Single *z*-sections were collected or 3D images generated from 25-30 (0.8 μm thick) *z*-sections using Zeiss LSM 3.0 software. Alternatively, images (Fig. 4A) were collected using a Leica TCS SP MP confocal microscope (Leica, Wetzlar, Germany) with a 63×/1.4 oil immersion planapochromat objective. Alexa Fluor 488 fluorescence was excited using a 488 nm excitation line from an argon/krypton laser and the emission window was set at 492-560 nm.

CD95 DISC analysis

1.5×10^7 Jurkat cells and 1×10^7 H9 cells per sample were left untreated or pre-treated with 20 μM cytD (1 hour), 20 μM zIETD (1 hour) or 20 μM Y-27632 (1 hour) at 37°C. Cells were pelleted and resuspended in 1 ml pre-warmed RPMI-FCS. To stimulate CD95, 0.5 μg αCD95 (CH11) was added to the cell suspension. Cells were incubated in a 37°C water bath for the indicated time periods and the reaction was stopped by transferring the cells onto ice. Cells were pelleted, washed with ice-cold PBS and lysed in 250 μl lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 0.2% Nonidet P-40, 2 mM sodium orthovanadate and complete protease inhibitor cocktail; Roche, Basel, Switzerland) for 30 minutes on ice. Cell debris was removed by centrifugation at 5000 ***g*** for 10 minutes at 4°C. Rabbit αCD95 (1:100; C-20; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the lysates and incubated for 1 hour prior to immunoprecipitation with BSA-blocked protein G (5 μl; Amersham Biosciences, Buckinghamshire, UK) and protein A (5 μl; Sigma) beads for 3 hours at 4°C. Beads were washed six times in lysis buffer and finally resuspended in Laemmli sample buffer and boiled for 5 minutes. Proteins from cell lysates and immunoprecipitation (IP) samples were resolved by 12.5% or 10% SDS-polyacrylamide gel electrophoresis. Western blotting was performed using anti-caspase-8 [C15; a kind gift from Peter H. Krammer (Scaffidi et al., 1997)].

Detection of apoptosis

Analysis of caspase-3 activation

Untreated and treated samples were labeled with phycoerythrin (PE)-conjugated monoclonal active caspase-3 antibody apoptosis kit 1 (BD Pharmingen, Franklin Lakes, NJ, USA) for flow cytometry, according to the manufacturer's protocol. Briefly, cells were washed twice in PBS, fixed and permeabilized using Cytoperm/Cytofix solution for 20 minutes on ice and subsequently labeled with PE-conjugated active-caspase-3 monoclonal antibody in Perm/Wash solution for 30 minutes on ice. Cells were washed with Perm/Wash solution and were finally analyzed by flow cytometry (FACScan; BD Pharmingen).

DAPI labeling for detection of nuclear morphology

To distinguish between normal and apoptotic cells, treated and untreated cells were fixed in 3% PFA. Then, cytospin preparations were made and mounted in Vectashield mounting medium containing DAPI (Vector Laboratories). The labeled cells were viewed under a Leica DMRE epifluorescence microscope (Leica) equipped with a Fluotar 100×/1.3 oil PH3 objective. Images were captured using a Hamamatsu Photonics C4742-95 ORCA CCD camera (Hamamatsu, Japan).

Rho activation assay

Rho activation was analyzed by using a commercial assay (Upstate, NY, USA), according to the manufacturer's protocol, with some minor modifications. 1×10^7 cells were stimulated as indicated with 0.5 μg/ml αCD95 (CH11) in serum-free RPMI. The cells were then transferred onto ice and were washed twice with ice-cold TBS. Cells were lysed in 500 μl ice-cold Mg^{2+} lysis/wash buffer (MLB; 25 mM Hepes pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 10 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, complete protease inhibitor cocktail, 25 mM NaF, 1 mM sodium orthovanadate). After removal of insoluble material by centrifugation, the lysates were incubated for 1 hour at 4°C with 50 μl of Rhotekin RBD-agarose slurry, washed three times in MLB and were then boiled in Laemmli sample buffer containing DTT. Proteins from lysates and IP samples were then resolved by 15% SDS-polyacrylamide gel electrophoresis. Western blot was performed using anti-RhoA (Santa Cruz).

Analysis of ligand-receptor interactions

Raji cells were transiently transfected with CD95L-GFP by electroporation (220 V, 975 μF). After 36 hours, the transfected cells were incubated with an equal amount of Jurkat cells in 100 μl RPMI for 20 minutes and were then settled on polylysine-coated coverslips for an additional 10 minutes. The cells were washed once in PBS and then fixed with ice-cold methanol for 5 minutes. After blocking in 1% BSA/PBS the cells were labeled with αCD95-Cy3 (ICO-160). After mounting in 80% glycerol, the cells were viewed under a Leica fluorescence microscope.

Western blotting

Immunoblotting was performed by lysing cells in Laemmli sample buffer and then resolving the proteins using 10% or 12.5% SDS-PAGE. The separated proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany), probed with the specific antibodies to caspase-8 or RhoA, followed by coupling to the appropriate HRP-conjugated secondary antibodies and visualized with the ECL system (Amersham).

Results

Capping of CD95 is mediated by different mechanisms in type I and type II cells

The capping of CD95 has recently been discovered as one of the first events to occur after crosslinking of the receptor (Cremesti et al., 2001; Grassme et al., 2001b). To further clarify the role of this event, we explored the mechanisms responsible for CD95 capping in the type I cell H9 and the type II cell Jurkat. Cells were first labeled by incubating the cells with agonistic αCD95 antibody for 30 minutes followed by incubation with a secondary anti-mouse fluorescent antibody on ice. The labeled cells were then transferred to a 37°C water bath to induce capping. The cells representing the 0 minute time point (0') were kept on ice throughout the experiment. We found that CD95 capping occurred rapidly after stimulation with the agonistic αCD95 antibody in both cell types. Already after 2 minutes of stimulation, capping of CD95 was clearly observed, using confocal microscopy (Fig. 1A,B). The capping was not a consequence of an non-specific antibody response, since similar results were obtained with directly labeled agonistic CD95 antibodies (data not shown). Recently, actin was shown to participate in the organization and signaling of CD95 (Parlato et al., 2000). As it has been indicated that inhibition of actin polymerization could inhibit both CD95 capping and subsequent apoptosis, we wanted to elucidate the role of actin microfilaments in CD95 capping in type I and type II cells. To this end, we incubated H9 and Jurkat cells with cytochalasin D (cytD), a specific inhibitor of actin polymerization, for 1 hour prior to CD95 engagement. We

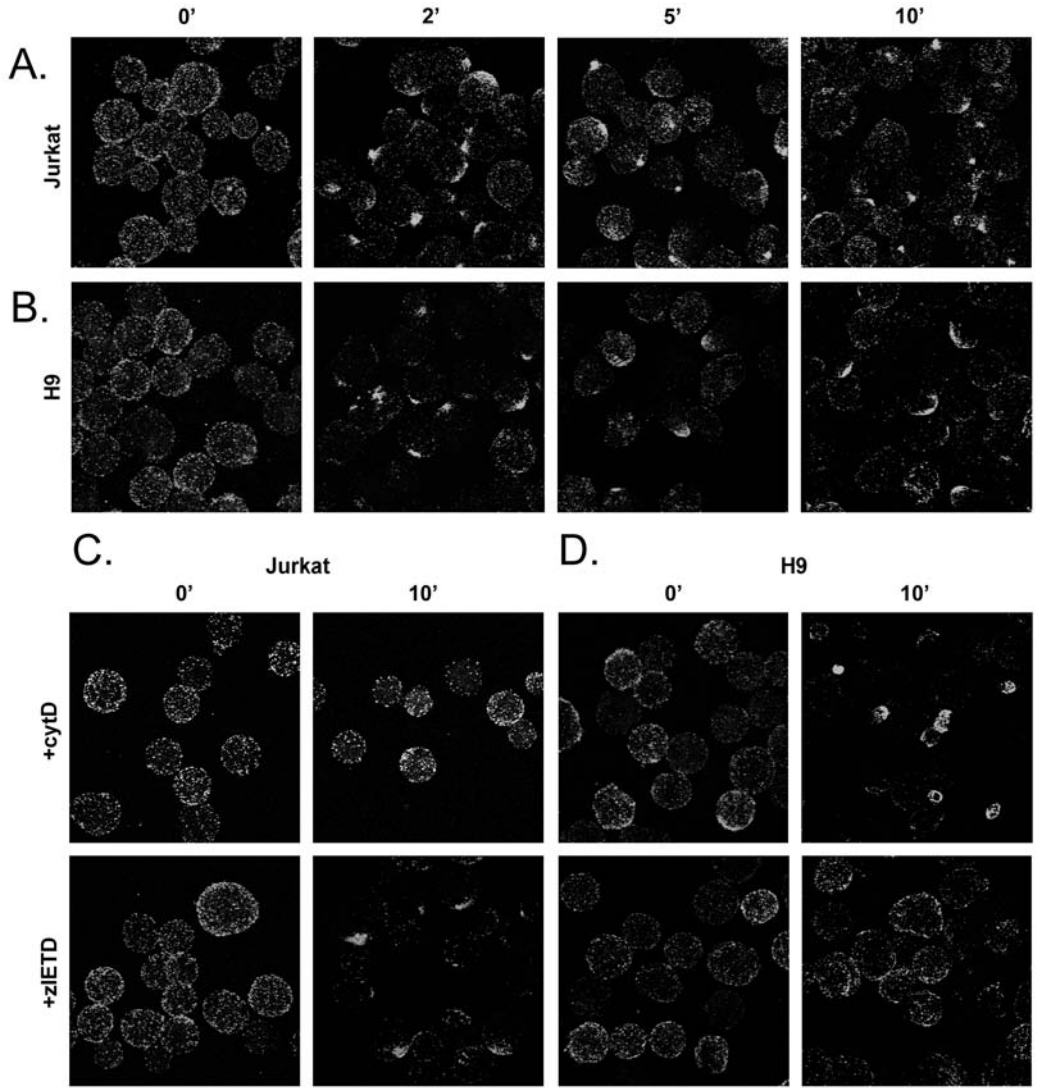

Fig. 1. CD95 capping occurs by different mechanisms dependent on cell type. (A) Jurkat and (B) H9 cells were labeled with αCD95 for 30 minutes on ice. After washing, the cells were incubated with a fluorescent secondary antibody on ice. The labeled cells were then stimulated by incubation at 37°C for the indicated time (0-10 minutes) to induce CD95 capping. In C,D, Jurkat and H9 cells were preincubated with cytD or zIETD for 1 hour to determine the requirement for actin or caspase-8 activation for CD95 capping. Fixed samples were then analyzed by confocal laser scanning microscopy. Maximum projections were generated from *z*-sections and representative fields from at least three separate experiments are shown.

found that cytD efficiently prevented capping of CD95 in Jurkat type II cells (Fig. 1C), but, interestingly, not in H9 type I cells (Fig. 1D), demonstrating cell type-specific requirements for actin reorganization during CD95 capping.

To determine whether some of the presumed initial CD95-mediated signals could be responsible for the capping of CD95, we analyzed if caspase-8 activation is required for the capping. To explore the importance of caspase-8 activation in the initiation of the capping process, we used the selective caspase-8 inhibitor zIETD (Medema et al., 1997). Pre-incubation of the cells with zIETD for 1 hour abolished the capping of CD95 only in the type I cell H9 (Fig. 1D). In Jurkat cells, the inhibition of caspase-8 did not affect CD95 capping (Fig. 1D). Again there was a striking difference between type I and type II cells, indicating that the mechanisms behind CD95 capping are cell type-specific.

CD95 capping does not correlate with the induction of apoptosis

We further wanted to examine whether the capping itself, or the inhibition of capping would affect the apoptosis signaling in the two different cell models. To this end, we first pre-treated the cells with the pharmacological inhibitors cytD and zIETD and then activated CD95 by adding αCD95. To detect the level of apoptosis, we labeled the cells with antibodies that recognize the activated cleaved caspase-3. The labeled cells were then analyzed by flow cytometry. The cells that were treated with αCD95 alone showed clear apoptosis after the indicated time of stimulation (Fig. 2A). The level of apoptosis correlated well to the CD95 capping seen at the early phases of CD95 triggering. When the cells were pre-treated with cytD, the capping of CD95 was abolished only in type II Jurkat cells, as indicated in Fig. 1C. Surprisingly, when we analyzed these cells for apoptosis, we found no inhibitory effect on apoptosis following cytD treatment (Fig. 2A). In the type I cell H9, in which cytD did not inhibit CD95 capping, it also did not inhibit apoptosis (Fig. 2B). To further clarify that the observed caspase-3 activity in Jurkat cells, in the presence of cytD, reflected apoptosis, we labeled the same samples with DAPI to assess their nuclear morphology. Apoptotic nuclear chromatin condensation was clearly observed in the presence of cytD when CD95 capping was inhibited (Fig. 1D, Fig. 2C). Our results further suggest that actin reorganization is not required for apoptosis. Since we observed massive CD95-mediated apoptosis in Jurkat cells that had lost the CD95 capping ability because of the disturbed actin reorganization, we conclude that capping is not a necessary event for the induction of apoptosis.

To further investigate the CD95-mediated apoptosis pathway under the same conditions, we analyzed the overall cleavage

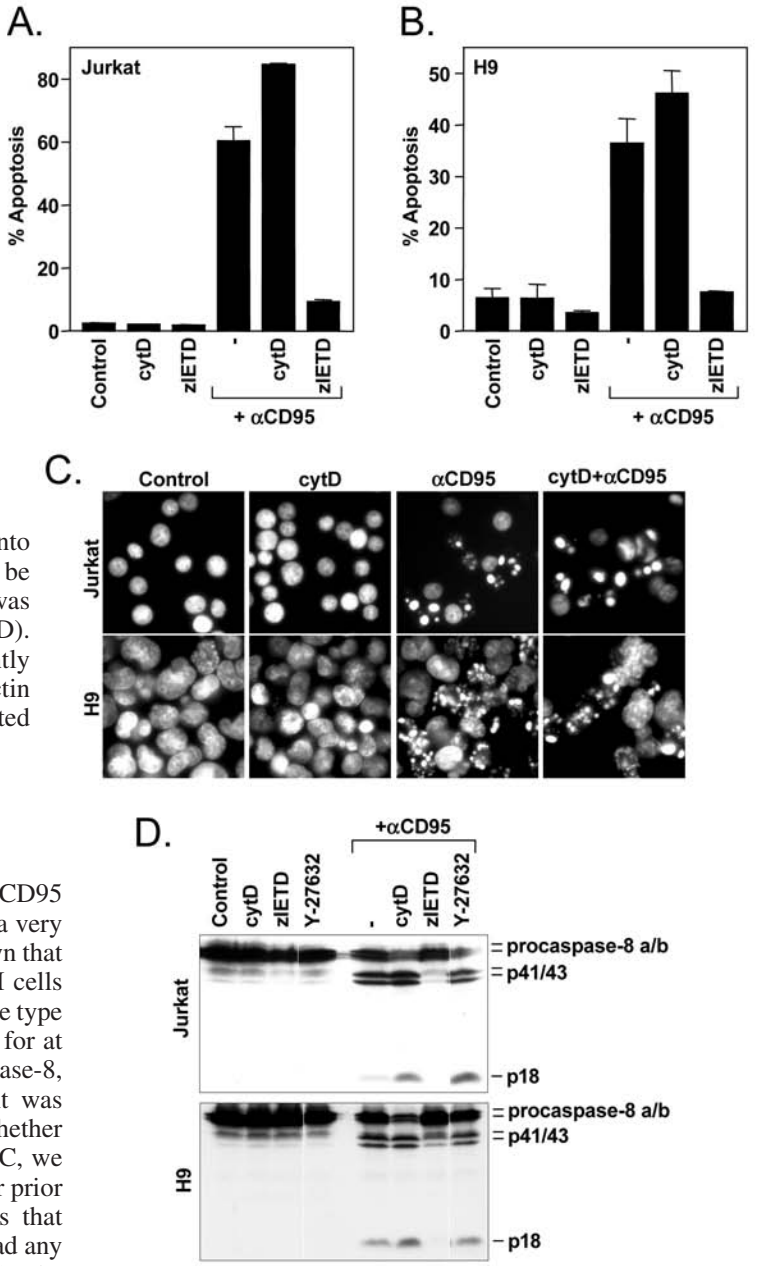

Fig. 2. CD95 capping does not correlate with induction of apoptosis. (A) Jurkat and (B) H9 cells were pre-treated for 1 hour with cytD or zIETD and were then incubated with 200 ng/ml αCD95 for 2 hours (Jurkat) or 6 hours (H9) to induce apoptosis. Cells were then labeled and analyzed for the presence of activated caspase-3, by flow cytometry. The data are mean values (±s.e.m.) from a minimum of three separate experiments. (C) Nuclear morphology of CytD- and αCD95-treated cells was further analyzed by DAPI labeling. Typical apoptotic cells with condensed chromatin can be seen in the αCD95 treated samples. CytD treatment did not inhibit the nuclear alterations associated with apoptosis. (D) Cells were treated as in A. and were then assessed for overall cleavage of caspase-8 by immunoblotting. Overall caspase-8 cleavage does not correlate with the capping of CD95 (Fig. 1C,D). Representative immunoblots from two separate experiments are shown.

of caspase-8. When caspase-8 is activated, it is cleaved into intermediate p41/43 and p18 fragments, which can be detected by western blotting. Cleavage of caspase-8 was clearly visible when CD95 capping was inhibited (Fig. 2D). The overall cleavage of caspase-8 was even slightly enhanced in the cytD-treated cells in which actin polymerization was inhibited, correlating with the elevated caspase-3 cleavage (Fig. 2A).

CD95 capping is not required for assembly of the DISC

To further dissect possible pathways initiated by CD95 capping, we analyzed the assembly of the CD95 DISC, a very proximal event after receptor triggering. It has been shown that type I cells form higher quantities of DISC than type II cells upon receptor ligation (Scaffidi et al., 1999). Indeed, in the type I cell H9, the DISC assembled faster and was sustained for at least 15 minutes as judged by the recruitment of caspase-8, while in the type II cell Jurkat, caspase-8 recruitment was slower and maximal at 5 minutes (Fig. 3A). To find out whether CD95 capping correlated with the formation of the DISC, we pre-treated the cells with either cytD or zIETD for 1 hour prior to activation of CD95 for 10 minutes. Fig. 3B shows that neither inhibition of actin polymerization or caspase-8 had any significant effects on the assembly of the CD95 DISC. The DISC was also assembled during conditions when CD95 capping was inhibited (cytD-treated Jurkat and zIETD-treated H9 cells; Fig. 1C,D). These results clearly show that CD95 capping and actin reorganization are not required for DISC assembly in either of the cell types, confirming that CD95 capping is not required for initiation of apoptosis.

CD95 mediates rapid actin reorganization and cocapping of actin and CD95

As triggering of several immune receptors mediates reorganization of microfilaments required for capping, we wanted to test whether CD95 triggering had any affect on the reorganization of actin. CD95 has previously been shown to associate with the actin cytoskeleton indirectly through the cytoskeletal linker protein ezrin (Parlato et al., 2000) and CD95 capping was suggested to be actin dependent (Algeciras-Schimnich et al., 2002). To analyze the reorganization of actin upon CD95 engagement, we labeled Jurkat cells with Alexa Fluor 546 phalloidin to identify filamentous actin (F-actin). We found that in control cells, F-actin was evenly distributed around the cells. When CD95 was activated, F-actin was rapidly reorganized and localized to one side of the cells, giving the cells a polarized phenotype (Fig. 4A). To analyze the relationship between actin reorganization and capping of CD95, we labeled CD95 together with F-actin. We found that in control cells, CD95 did not colocalize with F-actin and was instead localized in distinct patches around the cell membrane (Fig. 4B). After CD95 triggering, when capping was induced, we found a strong colocalization of F-actin and CD95 on one side of the cell. When actin polymerization was disrupted by cytD treatment, CD95 capping and colocalization with F-actin was abolished only in type II Jurkat cells. CD95 cocapping with actin in the type I cell

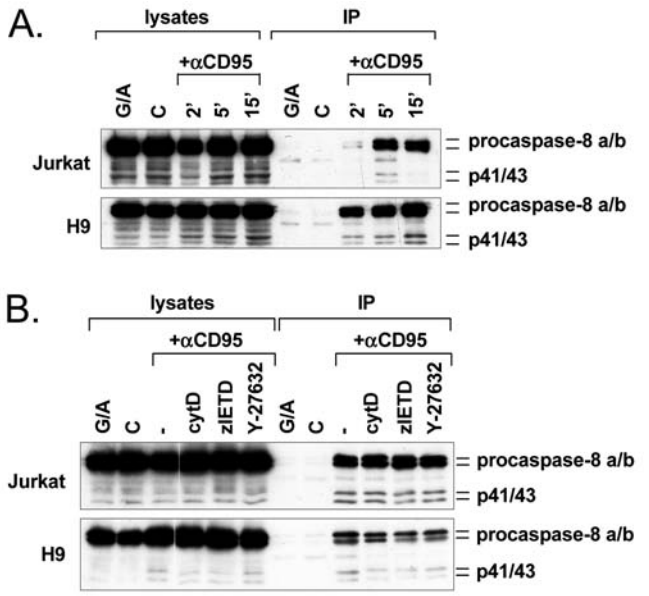

Fig. 3. Assembly of the CD95 DISC is independent of CD95 capping. (A) Jurkat and H9 cells were assessed for the presence of caspase-8 in the CD95 DISC by immunoprecipitation of CD95 after stimulation with αCD95 for the indicated time. (B) Jurkat and H9 cells were pre-treated as in Fig. 2A and were then subjected to immunoprecipitation of CD95 after stimulation with αCD95 for 10 minutes. Cell lysates and IP samples were analyzed for the presence of caspase-8. The treatments that abolished CD95 capping did not inhibit recruitment of caspase-8 and assembly of the CD95 DISC. Representative immunoblots from two separate experiments are shown.

H9 was even more intense in the presence of cytD (Fig. 1D and Fig. 4B). In all treatments in which CD95 capping occurred, F-actin colocalized with CD95 but not otherwise. These results led us to the conclusion that a functional actin cytoskeleton is important for the initiation of CD95 capping only in the type II cell Jurkat, but not in the type I cell line H9. CD95 capping always occurred together with capping of F-actin, resulting in polarized cells. Given the previous results using the actin organizer ezrin as a determinant for CD95 sensitivity (Parlato et al., 2000), we tested whether the different levels of ezrin could explain the different capping behaviors between a type I and type II cell, but could not find any significant differences in ezrin expression in Jurkat versus H9 cells (data not shown). Taken together, these results indicate that signaling mediated by CD95 induces rapid actin cytoskeletal reorganization, which is not required for the induction of apoptosis in Jurkat type II cells (Fig. 2A,B, Fig. 4; see also Fig. 8A and corresponding section in the discussion).

CD95 capping occurs concomitantly with aggregation of lipid rafts

Lipid rafts, rich in glycosphingolipids and cholesterol, have recently been shown to be involved in CD95 receptor organization and signaling (Gajate and Mollinedo, 2001; Grassme et al., 2001a). Therefore, we wanted to see whether the CD95 caps containing F-actin also included important components of lipid rafts. The ganglioside GM1 is a typical marker of lipid rafts and can easily be detected by labeling with fluorochrome-conjugated β-subunit of cholera toxin (CTxB). To detect lipid rafts, Jurkat cells were labeled with CTxB and were then stimulated with αCD95 for 10 minutes to induce capping. The cells were analyzed by fluorescence microscopy to determine the localization of GM1 in the cell membrane. Fig. 5A clearly shows that αCD95 stimulation rapidly mediated aggregation of lipid rafts from an even distribution around the cell membrane into a polarized pattern on one side of the cells in the same manner as F-actin (see Fig. 4A). To analyze whether CD95 colocalizes with lipid rafts, we labeled the cells for both CD95 and GM1. In control cells, the GM1-containing lipid rafts were located in patches evenly distributed around the membrane, separate from CD95 (Fig. 5B). After CD95 triggering, the labeling of GM1 showed a high degree of colocalization with CD95 within the caps, resembling the pattern seen in the F-actin-labeled cells (Fig. 5B,C). In the cells where CD95 capping was inhibited, we could not find any colocalization of CD95 and lipid rafts. Coaggregation of lipid rafts has been shown to occur with several receptors involved in immune regulation, such as the T cell receptor in immune synapses (for a review, see Alonso and Millan, 2001). Similar to actin reorganization, the localization of CD95 to lipid raft aggregates after receptor triggering does not seem to be important for apoptotic signaling, as cytD-mediated inhibition of lipid raft aggregation did not inhibit apoptosis and could occur in the absence of apoptosis (Fig. 5, Fig. 2A,B). Colocalization coincidenced with CD95 capping in both Jurkat and H9 cells, although the intensity of lipid raft aggregation was less prominent in the H9 cells. Therefore, the CD95-mediated aggregation of lipid rafts in Jurkat cells seems to be dependent on reorganization of actin, as cytD abolished the aggregation. It is interesting that the lipid raft aggregation in H9 cells occurred even when a functional actin structure was disrupted by cytD treatment. The aggregation of lipid rafts in H9 cells could therefore be regulated by other mechanisms, as F-actin reorganization was also not required for CD95 capping in these cells.

CD95 capping, but not apoptosis, is ROCK dependent

Since we found that CD95 capping can be regulated by the actin cytoskeleton, we wanted to see if ROCK, an established regulator of microfilament organization and many immune cell functions, could affect the capping.

It has been shown that capping of some immune receptors is ROCK dependent (Tharaux et al., 2003). Therefore, we investigated whether perturbation of ROCK signaling was able to modulate the rapid capping of CD95. Pre-treatment of Jurkat and H9 cells with the specific ROCK inhibitor Y-27632 completely inhibited CD95 capping in both cell types (Fig. 6A). Since ROCK signaling mediates reorganization of the cytoskeleton, we also labeled F-actin in the cells. In cells treated with αCD95 alone, F-actin colocalized with the polarized CD95, but ROCK inhibition abrogated the colocalization (Fig. 6A). Rho-GTPases and their downstream effectors, such as ROCK, have been implicated in the regulation of apoptosis in a number of cellular systems (for a review, see Riento and Ridley, 2003). To find out how the inhibition of ROCK affected CD95-mediated apoptosis in Jurkat and H9 cells when CD95 capping was inhibited, we pre-

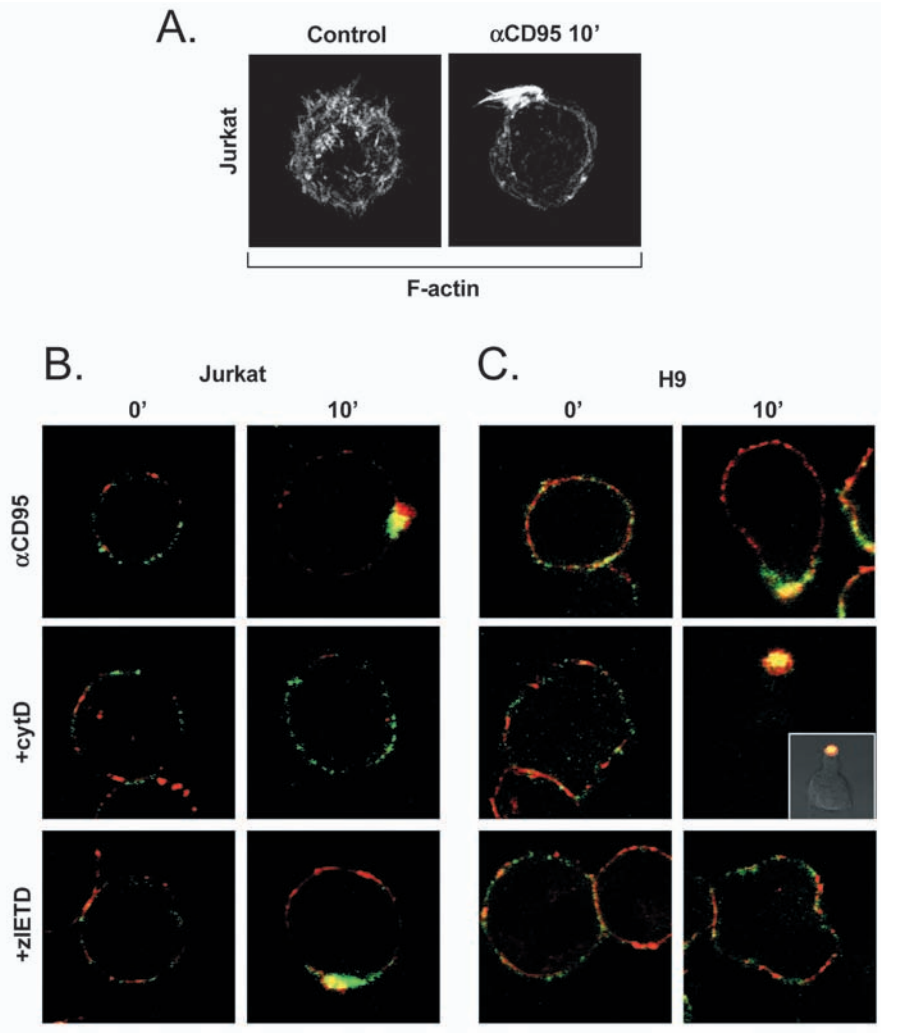

Fig. 4. Actin colocalizes with CD95 capping, giving the cells a polarized phenotype. (A) Jurkat cells were treated with αCD95 for 10 minutes at 37°C to stimulate the cells. Fixed cells were then labeled with Alexa Fluor 488 phalloidin to detect F-actin. Maximum projections from confocal *z*-sections were generated and representative cells from at least three separate experiments are shown. F-actin rapidly reorganizes to one pole of the cell upon CD95 activation. (B) Jurkat and (C) H9 cells were treated and labeled for CD95 (green) as in Fig. 1. The fixed and permeabilized cells were then further labeled with Alexa Fluor 546 phalloidin (red). Finally, single confocal *z*-sections were acquired. Overlay pictures of representative cells from at least three separate experiments are shown. CD95 colocalizes with F-actin only when CD95 capping is allowed (yellow).

treated the cells with Y-27632 and then incubated them with αCD95, and finally analyzed the activation of caspase-3 by flow cytometry. Although ROCK inhibition was clearly able to abrogate capping of CD95, the level of apoptosis was not suppressed (Fig. 6B). Interestingly, the number of apoptotic H9 cells was nearly doubled in the presence of Y-27632 even if Y-27632 alone did not induce apoptosis (Fig. 6B). The CD95-mediated overall caspase-8 activation was also higher in the presence of Y-27632 (Fig. 2D). These results indicate that ROCK signaling can provide survival signals, at least in the type I cell H9. To confirm that the cells were really apoptotic, we labeled the same samples with DAPI to assess their nuclear morphology. The DAPI labeling clearly showed that the cells treated with the Y-27632 and αCD95 had condensed chromatin and could therefore be considered apoptotic (Fig. 6C). We also analyzed the assembly of the CD95 DISC in the presence of Y-27632. Inhibition of ROCK did not abrogate caspase-8 recruitment to the DISC (Fig. 3B). Taken together, our results clearly show that CD95 capping is ROCK-dependent and uncoupled from the apoptotic machinery.

CD95 activates RhoA only in the type II cell Jurkat

As we found that ROCK was required for the capping of CD95, we further wanted to see if CD95 engagement resulted in activation of the small GTPase RhoA, an upstream activator of ROCK. For this purpose, we treated both Jurkat and H9 cells with αCD95 and pulled down RhoA-GTP to measure the amount of activated RhoA. As shown in Fig. 7, CD95 engagement resulted in rapid activation of RhoA after receptor triggering in Jurkat cells. CD95 was able to activate RhoA only in the type II cell Jurkat while Rho-GTP was absent in the type I cell H9. The levels of total RhoA in the lysates were not affected by CD95 engagement (Fig. 7). These results suggest that in Jurkat cells, RhoA activation upon CD95 engagement is a probable mechanism for the activation of its downstream substrate ROCK. To our knowledge, these results show for the first time that CD95 can activate RhoA. The αCD95-mediated activation of RhoA was not required for apoptosis as inhibition of RhoA was not able to interfere with apoptosis in Jurkat cells (data not shown). Taken together, these results show that RhoA is activated upon CD95 engagement only in the type II cell Jurkat, indicating that the ROCK activation in type I and type II cells takes place by distinct mechanisms.

Discussion

We have discovered that CD95 capping is not necessarily coupled to the apoptotic program, since apoptosis can occur without capping and capping can occur without apoptosis. We found that CD95 capping occurs concomitantly with polarization of F-actin and lipid raft aggregation. Both F-actin and lipid rafts rapidly colocalize with CD95 after receptor engagement. We also describe a novel function for ROCK in the death receptor signaling pathways. The capping of CD95 was completely dependent on ROCK, since inhibition of ROCK abrogated CD95 capping. Interestingly, inhibition of ROCK did not suppress, but instead lowered, the threshold for apoptosis induction. In addition, we show for the first time that in the type II cell Jurkat, CD95 activates the small GTPase

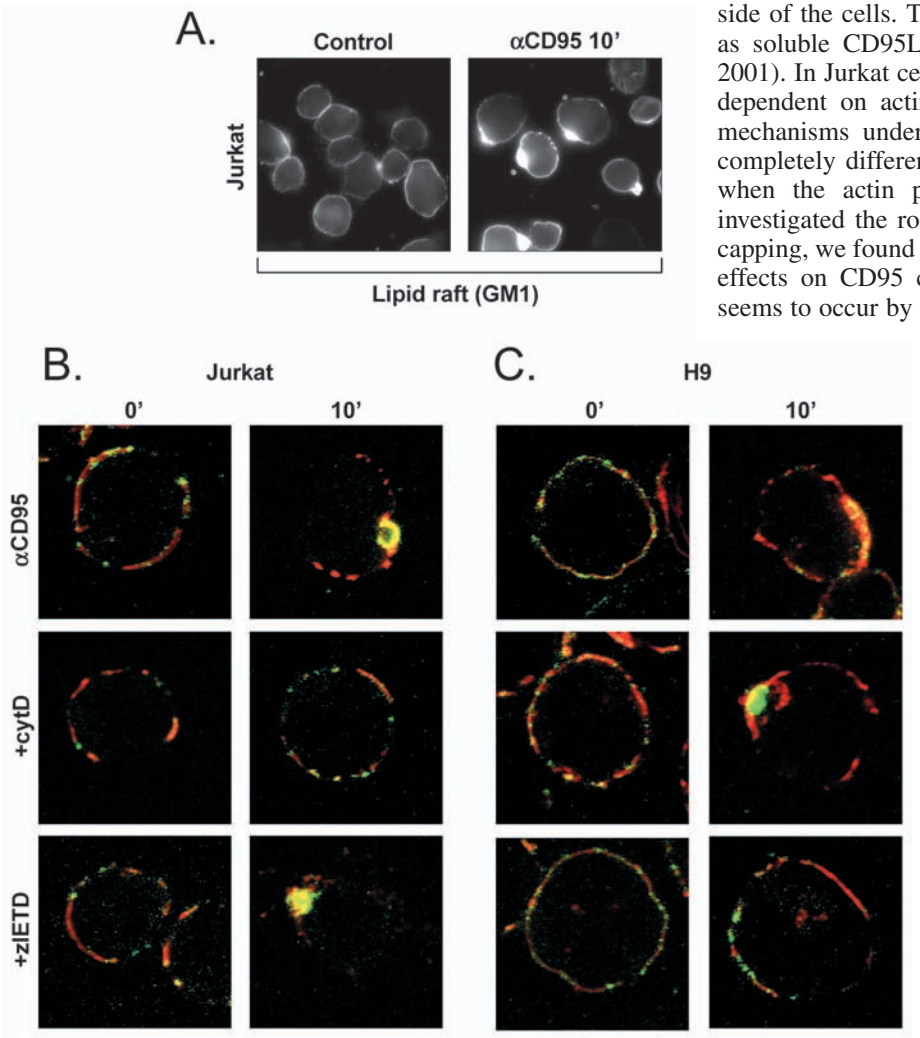

Fig. 5. CD95 stimulation induces rapid aggregation of lipid rafts. (A) Lipid rafts (GM1) in Jurkat cells were labeled with Alexa Fluor 555 CTxB and the cells were then treated with αCD95 for 10 minutes at 37°C. The fluorescence images show that αCD95 stimulation rapidly induced aggregation of the lipid rafts to one pole of the cells. (B) Jurkat and (C) H9 cells treated as in Fig. 3 were labeled with Alexa Fluor 555 CTxB (red) and αCD95 (green). Cells were kept on ice (0') or transferred to 37°C for 10 minutes (10') to induce capping. CD95 colocalizes with lipid rafts only when CD95 capping is allowed (yellow). Single confocal *z*-sections were acquired and overlay images of representative cells from three separate experiments are shown.

RhoA, upstream of ROCK. Recently, the role of CD95 in both T cell activation and proliferation has gained much attention and new non-apoptotic functions for CD95 are emerging. It is plausible that the ROCK-dependent CD95 capping could have a role in regulating such CD95-mediated non-apoptotic functions where cytoskeletal polarization is required.

CD95 capping is conducted by different mechanisms depending on cell type

As CD95 capping is one of the first events to occur after triggering of the CD95 receptor, we attempted to comprehensively explore and compare the mechanisms behind CD95 capping in type I H9 cells and type II Jurkat cells. Within minutes after CD95 engagement, the receptors capped at one side of the cells. The capping requires crosslinking of CD95, as soluble CD95L cannot induce capping (Cremesti et al., 2001). In Jurkat cells we found that the capping of CD95 was dependent on actin reorganization, but to our surprise, the mechanisms underlying capping in H9 cells seemed to be completely different, as CD95 capping was permissible even when the actin polymerization was disrupted. When we investigated the role of caspase-8 for the induction of CD95 capping, we found that this pathway also had cell type-specific effects on CD95 capping. Therefore, the capping of CD95 seems to occur by different mechanisms in type I and II cells. This is not in agreement with previous reports showing that CD95 capping is actin dependent only in type I cells (Algeciras-Schimnich et al., 2002). The observed differences could be due to the use of different inhibitors and experimental setup. A recent report supports our data from Jurkat cells by showing that cytD treatment inhibits capping in CEM cells, another type II cell line (Parlato et al., 2000).

CD95 capping and apoptosis

We wanted to see if CD95 capping correlated with the induction and propagation of apoptosis. To our surprise, in Jurkat cells, in which cytD inhibited CD95 capping, αCD95 was still able to induce apoptosis. We also found that CD95 capping could occur normally in type II Jurkat cells even in the absence of apoptosis induction when caspase-8 was inhibited. Furthermore, CD95 capping occurred in cells overexpressing FLIP and in resting human peripheral T cells, both cell types that were resistant to CD95-mediated apoptosis (data not shown). The fact that caspase-8 inhibition did not abrogate the CD95 capping could reflect the physiological state when T cells have upregulated FLIP levels. In type I H9 cells, CD95 capping seems to correlate quite well with the induction of apoptosis. However, we found that inhibition of ROCK-signaling also abrogated CD95 capping in these cells, without interfering with the induction of apoptosis. While most previous reports have implicated that there is a causal relationship between capping and apoptosis, the results presented here clearly show that CD95 capping is not required for the induction of apoptosis and, vice versa, that capping can occur without triggering the apoptotic program. In agreement with our findings, a recent study found CD95 capping in restimulated T cells that have constitutive caspase-8 activity but are resistant to apoptosis (Strauss et al., 2003).

After triggering of CD95, FADD and caspase-8 are rapidly recruited to the receptor and form the DISC. The assembly of the pro-apoptotic DISC has been shown to occur in lipid rafts following CD95 triggering (Hueber et al., 2002; Scheel-

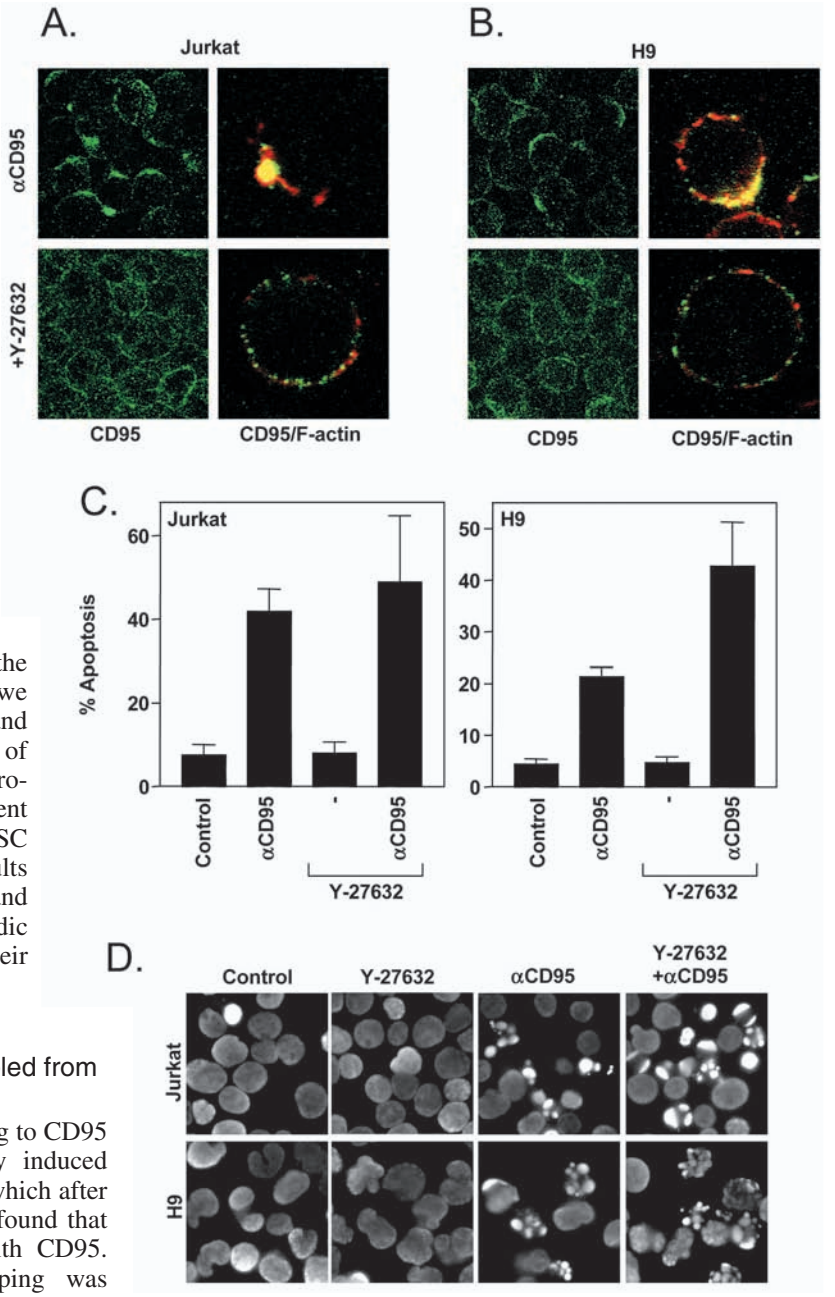

Fig. 6. CD95 capping but not apoptosis is ROCK dependent. (A) Jurkat and (B) H9 cells pre-treated for 1 hour with the ROCK inhibitor Y-27632 were labeled for CD95 alone (first panel; green) or together with F-actin (second panel; red) as in Fig. 1 and Fig. 4B,C. The cells that were pre-treated with Y-27632 did not show any capping of CD95. Maximum projections (left panels) or single overlay *z*-sections (right panels) from confocal images are shown. Inhibition of ROCK abolishes CD95 capping in both cell types. (C) Jurkat and H9 cells were pre-treated as in A and were then incubated with 200 ng/ml αCD95 for 2 hours (Jurkat) or 4 hours (H9) to induce apoptosis. Cells were then analyzed for activation of caspase-3 as in Fig. 2A,B. Inhibition of ROCK did not inhibit αCD95-mediated apoptosis. The data represent mean values (mean±s.e.m.) from a minimum of three separate experiments. (D) The nuclear morphology of samples from B were analyzed by DAPI labeling to confirm that the cells were apoptotic. Y-27632 did not inhibit nuclear alterations associated with apoptosis.

Toellner et al., 2002). When we analyzed whether the capping of CD95 could regulate DISC assembly, we could not find any correlation between capping and DISC assembly. This indicates that the capping of CD95 is uncoupled from the assembly of the pro-apoptotic DISC. This is not in agreement with a recent report showing that CD95 capping is required for DISC formation (Grassme et al., 2003). The different results could be due to the use of different cellular models and experimental setup as Grassme et al. used acidic sphingomyelinase-deficient B-lymphocytes in their experiments.

CD95 capping is ROCK-dependent and uncoupled from apoptosis signaling

When we investigated the sequence of events leading to CD95 capping, we found that CD95 triggering rapidly induced reorganization of the actin microfilament network, which after receptor triggering cocapped with CD95. We also found that lipid rafts rapidly aggregated and colocalized with CD95. These events occurred only when CD95 capping was permissible and they were clearly uncoupled from the apoptosis signaling. All of these processes were dependent on ROCK signaling. The ROCK-dependent capping was uncoupled from apoptosis signaling, since inhibition of ROCK did not inhibit apoptosis but abrogated all other events. Instead, we found that ROCK could be anti-apoptotic as ROCK-inhibition elevated apoptosis. We conclude that ROCK orchestrates the CD95 capping by allowing actin reorganization concomitantly with aggregation of lipid rafts. This model is plausible, since ROCK is known to be a potent modulator of actin reorganization (for a review see Narumiya et al., 1997) and can promote both lipid raft and receptor reorganization (Tharaux et al., 2003). Since we show that actin was not required for CD95 capping in the type I cell H9, other ROCK substrates such as vimentin could be involved in the CD95 capping in these cells (for a review, see Riento et al., 2003).

One possible way by which ROCK triggers CD95 capping, could be by regulating CD95 association with the actin cytoskeleton. It is known that the RhoA-ROCK pathway can phosphorylate proteins of the ezrin-radixin-moesin family, increasing their actin-membrane crosslinking activity (Matsui et al., 1998). Interestingly, CD95 binds to actin indirectly by associating with ezrin (Parlato et al., 2000). In this manner ROCK could mediate the interaction of CD95 with ezrin and thus subsequent receptor capping.

Possible roles of CD95 capping

In order to keep the homeostasis in tissues, it is important for cells to communicate with each other. One means of communication is receptor-ligand interactions. CD95 ligand

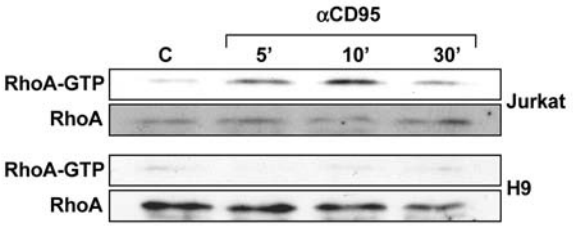

Fig. 7. CD95 activates RhoA only in the type II cell Jurkat. Cells treated with αCD95 for the indicated times were analyzed for activation of RhoA by Rho-GTP pull-down assay. Rho-GTP could be detected only in the αCD95-treated Jurkat cells, whereas it was absent in the αCD95-treated H9 cells. The level of total RhoA in the lysates did not change upon CD95 engagement. Representative immunoblots from two separate experiments with similar results are shown.

(CD95L)-CD95 interactions can in this sense signal apoptosis of unwanted cells. However, recent findings suggest that this interaction may also lead to several responses that are not coupled to apoptosis (for a review, see Wajant et al., 2003). Many of the functions associated with CD95 signaling are initiated by formation of immune synapses during cell-cell interactions, such as between T cells and antigen-presenting cells (e.g. dendritic cells) or cytotoxic T cells and their target cells. In this regard, the capping and subsequent polarization of CD95 could regulate the availability of CD95 at the synapse. Although we show here that CD95 capping does not directly correlate with apoptosis signaling in a single cell population, it could either promote cell-mediated killing by capping of the receptors towards the synapse or inhibit initiation of apoptosis by excluding CD95 from the synapse, thus minimizing the risk of a lethal hit by CD95L, analogously to what has been shown for other leukocyte receptors such as CD43 (Allenspach et al., 2001). Indeed, we found that CD95 can polarize towards a CD95L-expressing cell (Fig. 8A). It is interesting that CD95 capping can also be induced without receptor triggering (Gajate and Mollinedo, 2001). Recently, CD3 stimulation was found to induce CD95 translocation into lipid raft domains (Muppidi et al., 2004). Hence, signaling proteins at the synapse, such as the T-cell receptor or costimulatory receptors, could regulate the distribution and thus the availability of CD95 by mediating actin and lipid raft polarization (reviewed by Alonso and Millan, 2001). ROCK was found to be important for both lipid raft aggregation and F-actin polarization towards the synapse between cytotoxic T cells and their targets (Lou et al., 2001). In addition, costimulatory signals at immune synapses could mediate activation of ROCK thereby allowing CD95 capping and thus modulate susceptibility to CD95 triggering (Viola et al., 1999; Lou et al., 2001) (Fig. 8A,B). Inhibition of Rho-GTPases upstream of ROCK has been shown to inhibit the CD95-dependent killing of target cells (Subauste et al., 2000). We are currently investigating the role of CD95 capping in such regulatory functions (Fig. 8B).

Cytoskeletal and lipid raft reorganization and aggregation are important events during diverse functions in leukocytes (for a review, see Fais and Malorni, 2003). Cocapping of lipid rafts and receptors that regulate immune responses often occurs among costimulatory receptors (Viola et al., 1999; Yashiro-Ohtani et al., 2000) and integrins (Leitinger and Hogg, 2002). Hence, the lipid raft aggregation seems to be responsible for the delivery of membrane proteins to immune synapses, as has been shown during the assembly of supramolecular activation clusters in T cells (Jordan and Rodgers, 2003). The aggregation of lipid rafts requires actin reorganization in T cells (Villalba et al., 2001). In our model, the aggregation and delivery of lipid rafts seems to be conducted by CD95-mediated non-apoptotic signals that affect actin reorganization, at least in the type II Jurkat cells.

Our experiments do not explain whether CD95 itself activates ROCK or if a basal level of ROCK activity is required for CD95 capping. In addition to activation by RhoA, ROCK can be activated by direct caspase cleavage (Coleman et al., 2001; Ueda et al., 2001). In the type I cell H9, the CD95 capping was dependent on the activation of caspases (Fig. 1D). It is therefore probable that the caspase activation generated by CD95 in these cells could subsequently activate ROCK by cleavage, in contrast to the type II Jurkat cells, in which caspase inhibition did not impair CD95 capping. Instead exclusively in Jurkat cells, the RhoA mediator was mobilized, which is the probable reason for the activation of ROCK in these cells.

ROCK-dependent capping upon CD95 ligation could have an effect on the functions normally regulated by this kinase. A recent report suggests that CD95L bound to fibronectin induces rapid adhesion of T cells in an integrin-dependent fashion (Zanin-Zhorov et al., 2003). Adhesion of T cells normally requires capping and activation of integrins and other receptors. In this way CD95 capping could be responsible for the rapid adhesion seen in the Zanin-Zhorov et al. study. It has also been reported that both CD95L and αCD95 can be chemotactic and induce migration in leukocytes (Ottonello et al., 1999; Seino et al., 1998). Both adhesion and migration require cellular polarization and the CD95-mediated cellular polarization could in this way promote such functions during given conditions. ROCK was found to be important for detachment of the uropod in migrating leukocytes (Alblas et al., 2001) and for polarization and chemotaxis in T cells (Bardi et al., 2003). In this sense, the ROCK-dependent CD95 capping, actin reorganization, and their localization to structures such as the uropod could possibly regulate both leukocyte adhesion and migration. The CD95-mediated capping and induction of polarization could, therefore, have a role in such CD95-mediated non-apoptotic signaling where a polarized cell phenotype is required.

The different mechanisms behind CD95 capping in type I and type II cells could be a reflection of different activation states. It has been proposed that resting T cells that have low levels of CD95 surface expression are resistant to apoptosis and are therefore acting as type II cells. In contrast, activated T cells have upregulated CD95 resembling the type I cells (Schmitz et al., 2003). Therefore, the capping of CD95 could yield different results depending on the activation state of a single cell: in a resting T cell, CD95 capping could lead to non-apoptotic responses in a RhoA-ROCK-dependent manner (type II cell) and later in the activated T cell, CD95 signaling and capping would lead to apoptosis by a parallel ROCK-independent signaling pathway (type I cell) leading to resolution of an immune response (Fig. 8C).

In summary, the results presented here show that CD95 capping is ROCK-dependent and that the capping is not required for the induction of apoptosis by CD95. We also show

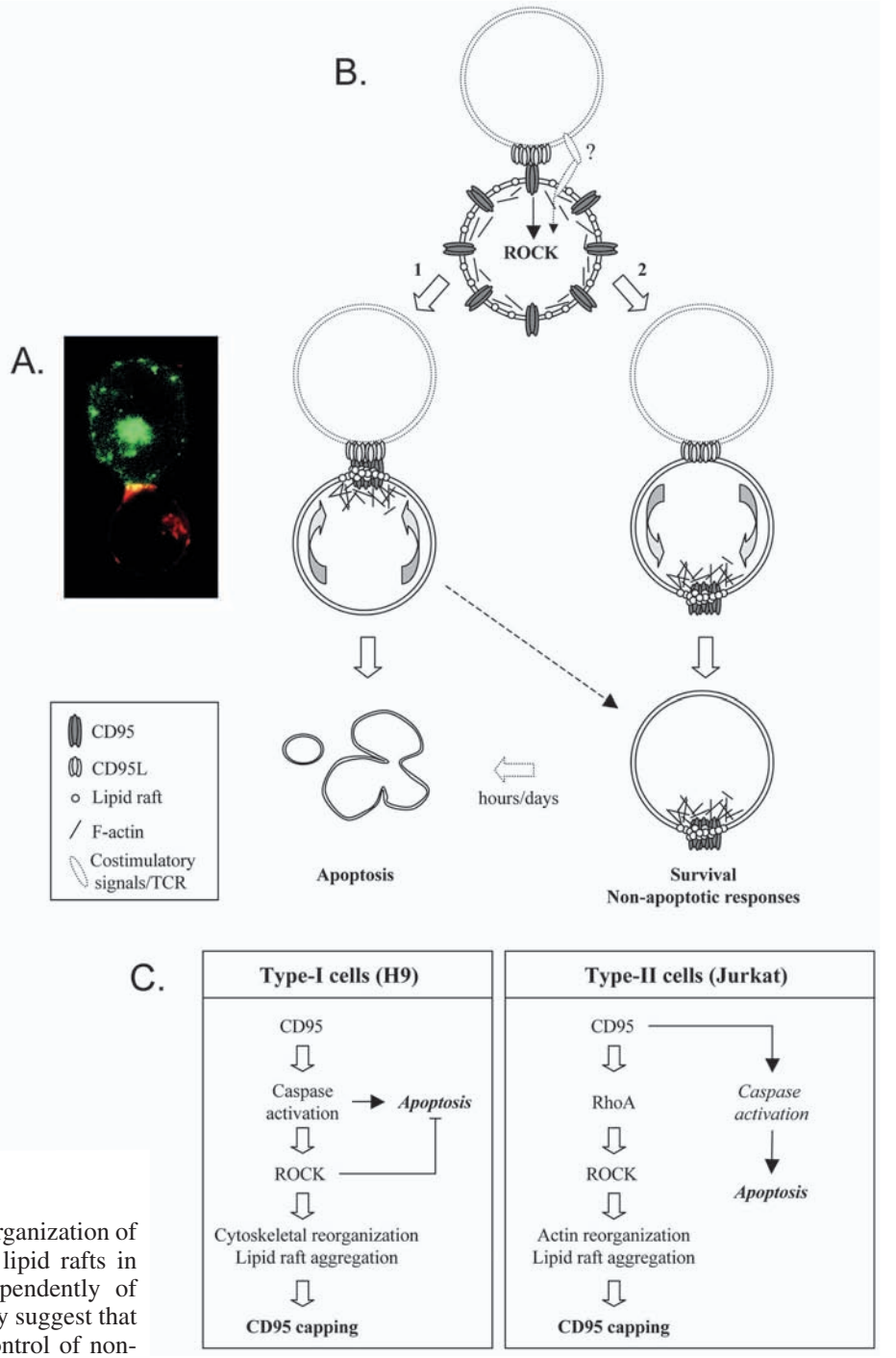

Fig. 8. (A) CD95 on the T-cell can polarize towards a CD95L-expressing cell. Raji cells transiently transfected with CD95L-GFP (green) were incubated with Jurkat cells. The mixture was then settled on polylysine-coated coverslips and was finally labeled for CD95 (red). Note that CD95 is polarized towards the CD95L-expressing cell. (B) CD95 capping could regulate the availability of CD95 at immunological synapses. In this model, CD95 triggering by CD95L expressed on an encountering cell leads to ROCK-dependent receptor capping with concomitant aggregation of lipid rafts and F-actin by a mechanism leading to polarization of the cell. When CD95 is polarized towards the synapse, the target cell would eventually die by apoptosis (1). It is also possible that CD95 would cap at the distal pole and the cell would live, unaffected by the CD95L expressed on the surface of the other cell (2). CD95 capping could also be mediated independently of its ligand by signals from the T cell receptor, costimulatory ligands, or anti-tumor drugs that would affect the sensitivity to cell-mediated killing. (C) CD95 capping is ROCK-dependent and uncoupled from apoptosis signaling. ROCK signaling promotes cytoskeletal reorganization and lipid raft aggregation and capping of CD95. In type I cells, ROCK activation could be mediated by caspase cleavage, which is required for both CD95 capping and apoptosis. In the type II cell CD95 engagement leads to RhoA activation, which in turn activates ROCK, actin reorganization, lipid raft aggregation and CD95 capping. The apoptotic pathway, involving caspase activation is separated from the CD95 capping pathway in the type II cell. However, also in these cells ROCK signaling is only required for CD95 capping and not apoptosis. In addition, ROCK signaling suppresses apoptosis in a feedback loop.

that early CD95 signaling induces rapid reorganization of the actin cytoskeleton and aggregation of lipid rafts in concert with the capping of CD95 independently of apoptosis induction. The results of this study suggest that CD95 capping could be involved in the control of non-apoptotic functions that need cellular polarization, such as T-cell activation, adhesion or migration in a ROCK-dependent fashion. The ability of CD95 to mediate both apoptotic and non-apoptotic signaling is an intriguing phenomenon that needs to be characterized in greater detail in order to understand disorders where dysregulated apoptosis is involved. Understanding the molecular mechanism behind the apoptosis-survival switch would give new insights in immune cell regulation.

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