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Recruitment of TNF Receptor 1 to Lipid Rafts Is Essential for TNFα-Mediated NF-κB Activation

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Summary

Engagement of TNF receptor 1 by TNFα activates the transcription factor NF-κB but can also induce apoptosis. Here we show that upon TNFα binding, TNFR1 translocates to cholesterol- and sphingolipid-enriched membrane microdomains, termed lipid rafts, where it associates with the Ser/Thr kinase RIP and the adaptor proteins TRADD and TRAF2, forming a signaling complex. In lipid rafts, TNFR1 and RIP are ubiquitylated. Furthermore, we provide evidence that translocation to lipid rafts precedes ubiquitylation, which leads to the degradation via the proteasome pathway. Interfering with lipid raft organization not only abolishes ubiquitylation but switches TNFα signaling from NF-κB activation to apoptosis. We suggest that lipid rafts are crucial for the outcome of TNFα-activated signaling pathways.

Introduction

Members of the tumor necrosis factor (TNF) family and their respective receptors play pivotal roles in the organization and function of the immune system. In addition, there is growing evidence that dysregulated TNF expression and/or signaling are implicated in various diseases, such as multiple sclerosis, Alzheimer's disease, and rheumatoid arthritis (Balkwill et al., 2000). A subfamily of TNF ligands, including TNFα and Fas ligand (FasL), are potent inducers of apoptosis (Aravind et al., 2001; Ashkenazi and Dixit, 1998; Baker and Reddy, 1998; Baud and Karin, 2001; Budd, 2001; Krammer, 2000; Locksley et al., 2001). In the case of TNFα, however, activation of transcription factors such as NF-κB, which drives the synthesis of a number of proinflammatory gene products (Baker and Reddy, 1998; Baud and Karin, 2001; Locksley et al., 2001) and antiapoptotic proteins (Micheau et al., 2001; Wang et al., 1998), is more commonly observed.

TNFα exerts its functions through two distinct receptors, TNFR1 (CD120a) and TNFR2 (CD120b). Activation of TNFR1 leads to the recruitment of the death domain (DD)-containing adaptor molecule TNFR1-associated death domain protein (TRADD), which serves as a platform to recruit additional mediators (Hsu et al., 1995, 1996). TRADD, in turn, binds the DD-containing Ser/Thr kinase receptor-interacting protein (RIP) and TNF-receptor-associated factor 2 (TRAF2). This TRADD-RIP-TRAF2 complex initiates the pathway leading to NF-κB activation (Devin et al., 2000; Hsu et al., 1996; Kelliher et al., 1998; Liu et al., 1996; Ting et al., 1996). TNFR1 can also recruit caspase-8 via TRADD and Fas-associated death domain protein (FADD/MORT1) to induce the signaling pathway leading to cell death (Hsu et al., 1996).

Binding of FasL induces receptor activation resulting in the recruitment of several signaling molecules to the DD present in its cytoplasmic region (Aravind et al., 2001; Ashkenazi and Dixit, 1998; Budd, 2001; Krammer, 2000). The DD of Fas (CD95) interacts with the DD of the adaptor FADD. This interaction unmasks the N-terminal death effector domain of FADD allowing the recruitment of procaspase-8 and caspase-10, which are autoproteolytically cleaved, leading to the assembly of the mature enzyme. Active caspase-8 and -10 subsequently cleave downstream caspases to initiate apoptosis (Aravind et al., 2001; Ashkenazi and Dixit, 1998; Budd, 2001; Krammer, 2000).

Binding of agonistic antibodies (Ab) or ligand to Fas or TNFR was demonstrated to cause receptor clustering at the cell surface (Dhein et al., 1992; Natoli et al., 1998). Such clustering has been observed for a variety of different receptors, and the fact that, upon stimulation, certain proteins involved in cell signaling are specifically recruited to or sequestered from microdomains has led to the idea that these lipid rafts serve as platforms for cellular signaling (Brown and London, 1998, 2000; Rietveld and Simons, 1998; Sedwick and Altman, 2002; Simons and Toomre, 2000). Lipid rafts are enriched in sphingolipids and cholesterol, which pack tightly against the saturated hydrocarbon chains of certain lipids (Brown and London, 2000). The role of microdomains in signal transduction emanating from members of the TNFR family has not been addressed until recently. Investigations, however, have produced ambiguous results. Fas was reported to be excluded from microdomains (Gajate and Mollinedo, 2001; Ko et al., 1999), and Fas signaling was not inhibited by the destruction of lipid rafts (Algeciras-Schimnich et al., 2002). In contrast, Fas was also shown to be constitutively associated with lipid rafts (Hueber et al., 2002) and to require ceramide-rich microdomains for signal transduction (Cremesti et al., 2001; Grassme et al., 2001a).

Similarly, discrepancies exist regarding the localization of TNFR1 in lipid rafts. TNFR1 was reported to localize exclusively in lipid rafts of unstimulated U937 cells. In addition, under lipoprotein-deficient conditions, TNFR1 surface expression and consequently TNFα-mediated apoptosis was reduced (Ko et al., 1999). In contrast, partitioning of TNFR1 in lipid rafts of fibroblasts was only observed upon TNFα activation (Veldman et al., 2001). Furthermore, TNFα stimulation was shown to in-

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crease a neutral sphingomyelinase activity in nonraft compartments. More recently, the DD of TNFR1 was reported to be required for partial localization of TNFR1 variants in microdomains of unstimulated HeLa cells (Cottin et al., 2002).

We therefore reinvestigated the role of lipid rafts in TNFα and FasL signaling. We show that lipid rafts play an essential role in TNFα-mediated NF-κB activation but not in FasL-induced cell death. Upon activation, TNFR1 but not Fas translocates to lipid rafts, where it binds to various signaling proteins, forming a receptor-induced signaling complex. We also show that the specific engagement of the receptor in lipid rafts induces ubiquitylation of both TNFR1 and RIP, providing evidence for a potential role of lipid rafts in the degradation of the signaling complex. Finally, we propose that the disruption of lipid rafts blocks NF-κB activation and hence sensitizes cells for apoptosis.

Results

TNFR1 but Not Fas Is Recruited to Lipid Rafts upon Engagement

In order to investigate whether lipid rafts are involved in TNFα- and FasL-mediated clustering of their receptors and initiation of the respective signal transduction pathway, the plasma membrane localization of endogenous TNFR1 and Fas was first examined on resting human fibrosarcoma HT1080 cells. Both TNFR1 and Fas were expressed at the cell surface, whereas TNFR2 was not detectable by FACS analysis (data not shown). The latter characteristic was further confirmed by the inability of TNFR2-specific Ab to inhibit the TNFα-mediated death of HT1080 cells rendered susceptible to apoptosis by expressing a mutated IκBα (data not shown and Micheau et al., 2001). Lipid rafts were isolated by discontinuous sucrose density gradients of Triton-X 100 cell lysates and were found in light fractions enriched in the lipid raft markers glycosphingolipid GM1, caveolin-1, and the Src kinase Fyn (Figure 1). Although a small amount of TNFR1 was found within lipid rafts, the large majority of the receptor was excluded from microdomains, together with the epidermal growth factor (EGF) receptor (Figure 1). Moreover, Fas was completely absent from lipid rafts (Figure 1).

Association of TNFR1 with lipid rafts was greatly increased upon receptor engagement (Figure 2A). A significant translocation was already observed as early as 2 min after TNFα addition and progressively declined after 10 min of stimulation down to its initial distribution in resting cells after 60 min. Early events occurring after TNFR1 activation are the recruitment of the adaptors TRADD and TRAF2, as well as the kinases RIP, IKKα, and IKKβ. All these proteins translocated to microdomains after 2 to 10 min of TNFα stimulation (Figure 2A). Lipid raft association of the receptor, as well as of the recruited signaling molecules, was transient and ceased after prolonged receptor triggering. Interestingly, raft translocation of TNFR1 and RIP was accompanied by modifications of both proteins as they migrated with higher apparent molecular weights on SDS-PAGE (Figure 2A). The fuzziness of the band obtained by the electrophoretic separation of the Triton X-100 soluble TNFR1

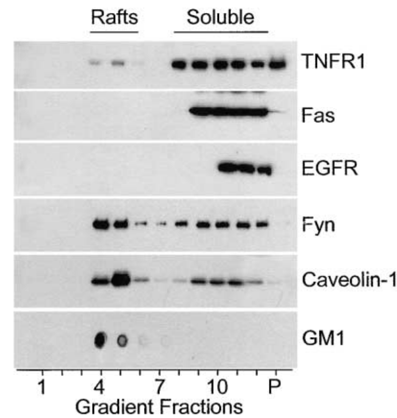

Figure 1. Constitutive Partitioning of TNFR1 in Lipid Rafts

HT1080 fibrosarcoma cells were lysed in 1% Triton X-100 and subjected to sucrose density gradient centrifugation to isolate lipid rafts. Proteins from equal volume of representative collected fractions were separated by SDS-PAGE and analyzed by Western blotting using specific Ab against TNFR1, Fas, EGFR, Fyn, and caveolin-1. To analyze the distribution of GM1, 2 μl of each fraction was dot blotted onto a nitrocellulose membrane and detected using CTx^{HRP}.

population was independent of TNFα triggering and was not observed upon shorter exposure of the same experiment (data not shown). In addition, the same unspecific fuzziness was also observed in the heavy fractions of the sucrose gradient depicted in Figure 1 upon longer exposure (data not shown). In contrast, FADD and caspase-8, which are involved in the apoptotic pathway, were not found in lipid rafts upon TNFα stimulation (Figure 2A). Finally, upon triggering of HT1080 cells with FasL, neither Fas nor any of the molecules involved in the early steps of the apoptotic signaling pathway, such as FADD and caspase-8, translocated to lipid rafts (Figure 2B). The absence of raft translocation was not due to inefficient Fas signals, as procaspase-8 was progressively processed after 20 min of FasL stimulation leading to apoptosis.

The TNFα Signaling Complex Is Predominantly Found in Lipid Rafts

Next, we investigated the TNFR1 and Fas signal transduction pathways by means of analysis of their signaling complexes. Additionally, we studied whether the signaling complex elicited by the engagement of endogenous TNFR1 occurred within or outside of microdomains. To this end, HT1080 cells were stimulated with Flag-tagged soluble TNFα, lipid rafts were isolated, and engaged receptors including the signaling complexes were immunoprecipitated using an anti-Flag Ab. As depicted in Figure 3A, the majority of engaged TNFR1 was found in lipid rafts after 2 min of stimulation, and lipid raft-associated TNFR1 was modified. In contrast, engaged TNFR1 molecules that were excluded from microdomains did not display such a modification. This difference was even more striking for RIP, which was maximally recruited to the raft-associated signaling complex after 2 min of TNFα stimulation where it was found almost exclusively in its modified forms (Figure 3A). The

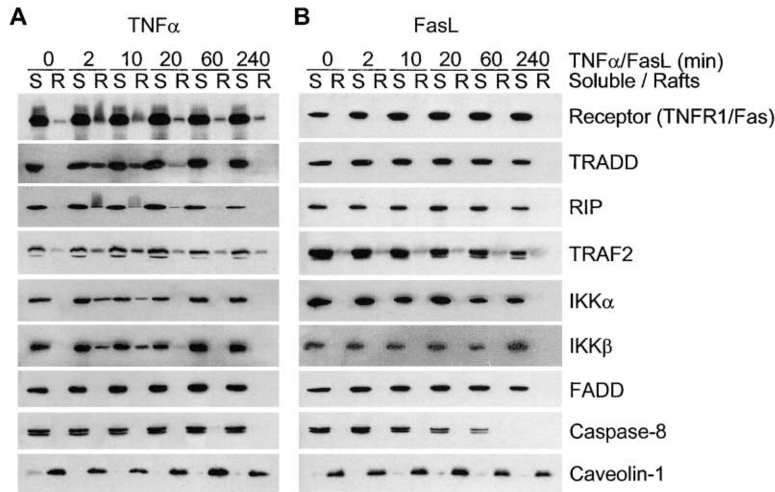

Figure 2. TNFα Stimulation Recruits Signaling Molecules to Lipid Rafts

HT1080 cells were stimulated with TNFα (A) or FasL (B) for the indicated time points, and Triton X-100 soluble (S) and insoluble lipid raft (R) fractions were isolated. Equal aliquots of the fractions were subjected to SDS-PAGE, and the protein distribution was assessed by Western blotting using specific Ab against TNFR1 (A) or Fas (B), TRADD, RIP, TRAF2, IKKα, IKKβ, FADD, caspase-8, and caveolin-1.

same pattern of modification was also observed upon TNFα stimulation of Jurkat T cells, myeloid leukemia U937 cells (data not shown), and HeLa cells (Chen et al., 2002; Zhang et al., 2000). Similarly, most of TRADD and TRAF2 recruited to the engaged TNFR1 were found in lipid rafts. Also, TRADD was modified upon TNFα stimulation, but modified species were found in both Triton X-100 soluble and insoluble fractions. Under these conditions, however, we failed to detect members of the IκBα kinase (IKK) complex with the engaged TNFR1, suggesting that these molecules, coimmunoprecipitated with the engaged TNFR1, are loosely bound to the complex and are released during the raft isolation procedure (data not shown and see below). Comparable results on the recruitment and modification of signaling molecules were obtained upon stimulation of HT1080 cells with an anti-TNFR1 Ab instead of TNFα (data not shown). These results provide evidence that, upon TNFR1 engagement, the signaling complex was formed and immediately translocated to lipid rafts, where both TNFR1 and RIP were modified. Consistent with previous data (Wallach et al., 1999), FADD and caspase-8 were not found in the membrane-associated TNFα signaling complex (Figure 3A). Moreover, after 2 to 10 min of TNFα stimulation, the signaling complex contained the lipid raft-resident caveolin-1 (Figure 3A), an observation that confirms the recent demonstration of the interaction between caveolin-1 and TRAF2 (Feng et al., 2001).

In contrast, FasL-mediated engagement of its receptor did not induce the translocation into lipid rafts of HT1080 cells of Fas or of any of the components of the Fas signaling complex (Figure 3B).

TNFR1 and RIP Are Ubiquitylated in Lipid Rafts

Protein modifications are important means of regulating their function, activity, or localization (Muller et al., 2001). Since ubiquitylation has been shown to play a role in receptor internalization and degradation, the modification observed for TNFR1 and RIP may be the addition of ubiquitin. To test this possibility, HT1080 cells were stimulated for 30 min with TNFα in the presence of the proteasome inhibitor lactacystin. This treatment resulted in a significant accumulation of modified TNFR1 and RIP in the raft-associated signaling complex, whereas, in the absence of lactacystin, the amount of modified species decreased after prolonged stimulation (Figure 4A). This result suggests that both proteins are ubiquitylated and subsequently degraded via the proteasome. To further verify whether engaged TNFR1 and recruited RIP were indeed ubiquitylated, we transfected HT1080 cells with HA-tagged ubiquitin. Western blot analysis of the immunoprecipitated TNFα signaling com-

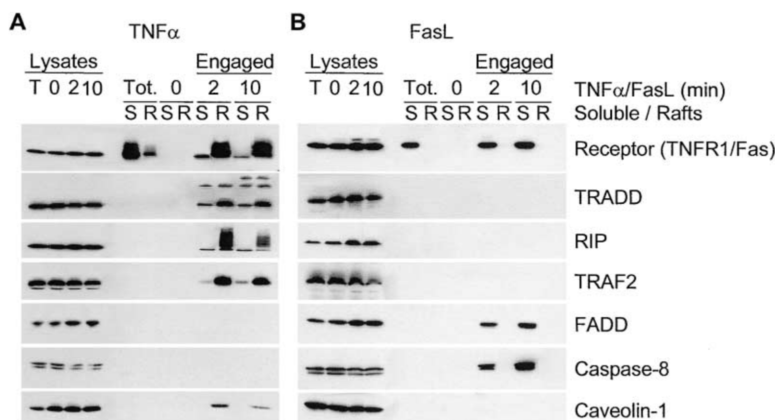

Figure 3. Accumulation of the TNFα-Induced Signaling Complex in Lipid Rafts

HT1080 cells were stimulated with Flag-tagged TNFα (A) or Flag-tagged FasL (B) for the indicated time points, and Triton X-100 soluble (S) and insoluble lipid raft (R) fractions were isolated. Engaged receptors and their signaling complexes were immunoprecipitated using anti-Flag Ab. For comparison, the total amount of receptor was immunoprecipitated by adding the ligand and anti-Flag Ab to the soluble and raft fractions of unstimulated cells. Immunoprecipitates and corresponding total cell lysates were subjected to SDS-PAGE and immunobloted using specific Ab.

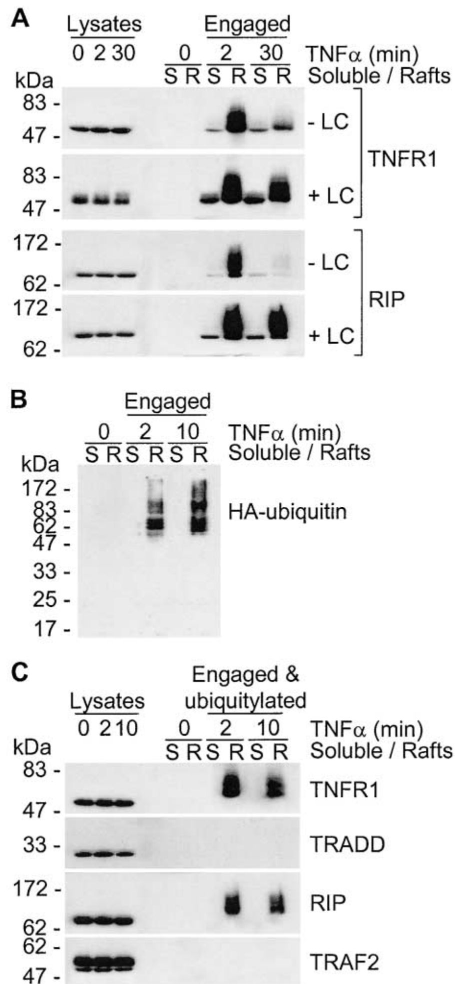

Figure 4. TNFα-Induced TNFR1 and RIP Ubiquitylation in Lipid Rafts

(A) HT1080 cells were incubated or not with the proteasome inhibitor lactacystin (LC) for 30 min, followed by stimulation with TNFα for 2 or 30 min. TNFα-induced signaling complexes from Triton X-100 soluble (S) and insoluble lipid raft (R) fractions were isolated and analyzed as in Figure 3.

(B) HT1080 cells were transiently transfected with HA-tagged ubiquitin, and TNFR1-associated signaling complexes were isolated 2 and 10 min after stimulation. Ubiquitylated proteins of TNFα-induced signaling complexes were revealed by immunoblotting using an anti-HA Ab.

(C) HT1080 cells were stimulated with TNFα, and the engaged receptors from Triton X-100 soluble (S) and insoluble lipid raft (R) fractions were immunoprecipitated using an anti-Flag Ab. The signaling complexes were dissociated by boiling in SDS, followed by immunoprecipitation using an anti-ubiquitin Ab. Engaged and ubiquitylated proteins were analyzed by Western blotting using Ab against TNFR1, TRADD, RIP, and TRAF2.

plex using anti-HA Ab revealed the presence of ubiquitylated proteins with apparent molecular weights corresponding to those of TNFR1 (55–70 kDa) and RIP (80–100 kDa) in the signaling complex of microdomains (Figure 4B). A more direct demonstration that the two ubiquitylated molecular species corresponded to TNFR1 and RIP was obtained by immunoprecipitation of the signaling complex by TNFα, followed by the dissociation of the complex by boiling in SDS, and immunoprecipitation of ubiquitylated molecules using an anti-ubiquitin Ab. Subsequent immunoblotting using specific Ab clearly revealed that both TNFR1 and RIP but not TRADD and TRAF2 were exclusively ubiquitylated in lipid rafts upon TNFα stimulation (Figure 4C).

Lipid Rafts Are Essential for Ubiquitylation, and Translocation Precedes Ubiquitylation

Since the TNFα signaling complex and ubiquitylation were found predominantly in lipid rafts, we investigated the role of microdomains in the formation of the signaling complex and in the protein modifications. To address this question, we treated HT1080 cells with dipalmitoyl phosphatidylethanolamine (DPPE), a phospholipid that we have previously shown to partition preferentially into lipid rafts, thus selectively blocking the recruitment of the T cell receptor signaling complex to microdomains without altering their structure (Legler et al., 2001). Thus, in HT1080 cells pretreated with DPPE prior to TNFα stimulation, the majority of the engaged receptor remained in the Triton X-100 soluble fraction (Figure 5A), demonstrating that DPPE blocked TNFα-induced translocation of TNFR1 to lipid rafts. Moreover, in DPPE-treated cells, only a reduced amount of RIP was recruited to the engaged receptor, and receptor-associated RIP was found predominantly in the nonraft fraction (Figure 5A), indicating that DPPE inhibited the translocation to lipid rafts of the entire signaling complex. Strikingly, both TNFR1 and RIP were not modified in DPPE-treated cells. In contrast, the lipid analog DOPE, which differs from DPPE only by having unsaturated oleic acid in place of saturated palmitic acid and which does not partition in lipid rafts (Legler et al., 2001), had no apparent effect on the recruitment of TNFR1 to lipid rafts, the assembly of the signaling complex, and on the ubiquitylation of TNFR1 and RIP (Figure 5A). These data support the notion that lipid rafts are critical for the stabilization/assembly of the TNFR1-associated signaling complex and that ubiquitylation takes place in lipid rafts.

To corroborate these findings, HT1080 cells were treated with methyl-β-cyclodextrin (MCD), which disrupts lipid rafts by specific cholesterol depletion (Harder et al., 1998; Janes et al., 1999). As shown in Figure 5B, in MCD-treated cells, TNFα triggering induced the recruitment of RIP to engaged TNFR1 in the nonraft fraction, and ubiquitylation of both TNFR1 and RIP was abolished. In order to test whether MCD treatment interfered with the association of other signaling molecules recruited to engaged TNFR1, cells were lysed in Brij78, a nonionic detergent with dispersing properties for both lipid raft- and nonraft-associated molecules. Specific immunoprecipitations of engaged TNFR1 from such cell lysates coimmunoprecipitated RIP, as well as IKKα and IKKβ. In contrast, MCD-mediated disruption of lipid rafts completely abolished the recruitment of the IKK complex to the triggered TNFR1 (Figure 5C). In control experiments designed to test the perturbing effect of MCD on the membrane raft structure, HT1080 cells were labeled with ^{14}C cholesterol, and the distribution of cholesterol was analyzed after lipid raft isolation. As shown in Figure 5D, MCD efficiently depleted cholesterol from the Triton X-100 insoluble lipid raft but not from the soluble fraction.

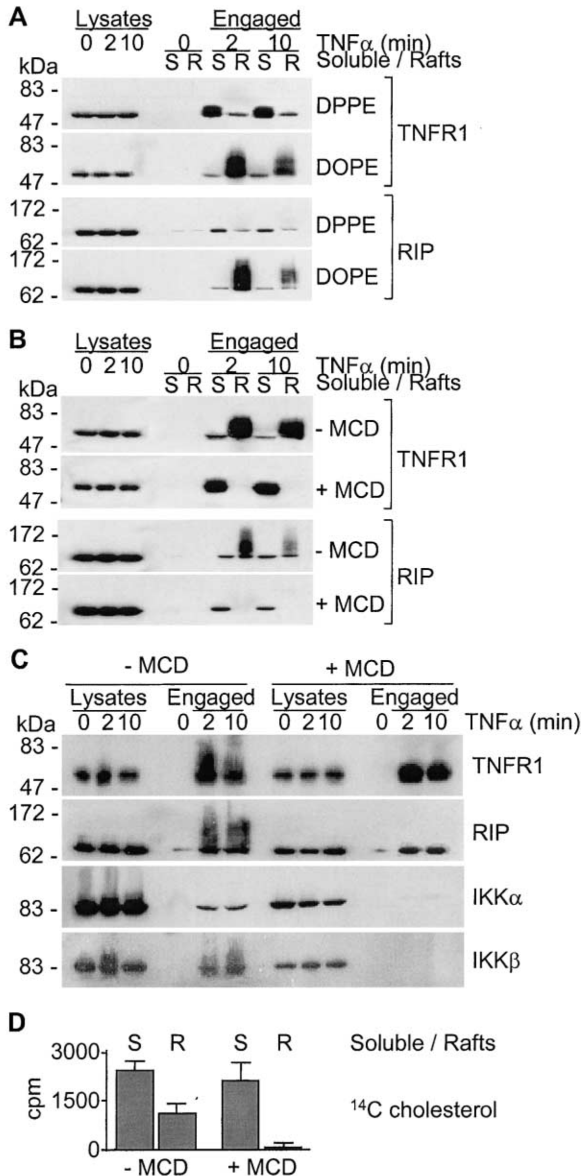

Figure 5. Lipid Rafts Are Essential for Ubiquitylation, and Translocation Precedes Ubiquitylation

(A) HT1080 cells were pretreated with DPPE or DOPE, respectively, for 30 min and stimulated with TNFα, and immunoprecipitated TNFR1 and RIP of the engaged signaling complex from Triton X-100 soluble (S) and insoluble lipid raft (R) fractions were analyzed by Western blotting.

(B) HT1080 cells were pretreated or not with MCD for 30 min prior to TNFα stimulation for 2 and 10 min. TNFR1 and RIP of the signaling complexes from Triton X-100 soluble and insoluble raft fractions were analyzed.

(C) HT1080 cells were treated or not with MCD, stimulated with TNFα for the indicated time points, and lysed in 0.5% Brij 78 for 2 hr followed by immunoprecipitation of the engaged TNFR1 using anti-Flag Ab; the engaged signaling complex was analyzed by Western blotting.

(D) HT1080 cells were labeled with ^{14}C cholesterol and treated or not with MCD, and the distribution of ^{14}C cholesterol was measured in Triton X-100 soluble and insoluble lipid raft fractions.

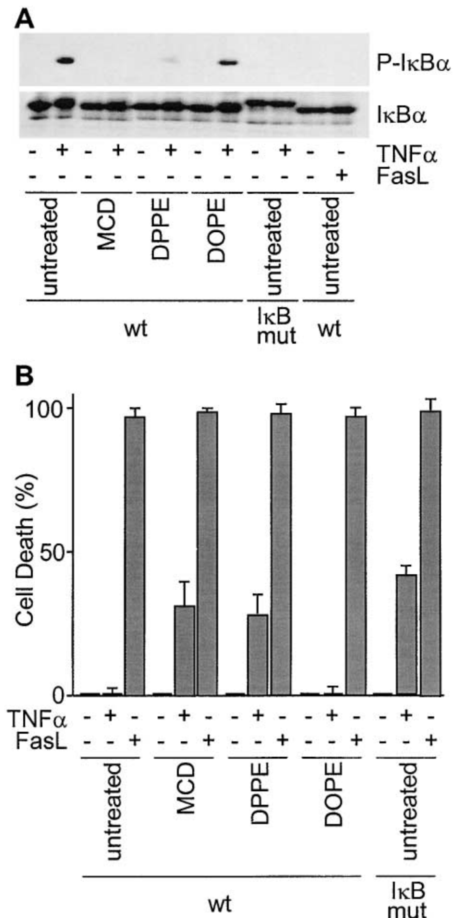

Figure 6. Blocking Lipid Rafts Inhibits TNFα-Mediated NF-κB Activation and Sensitizes for Apoptosis

(A) Wild-type (wt) or IκB mutant (IκB mut) HT1080 cells were pretreated or not with MCD, DPPE, or DOPE for 30 min, washed, and stimulated with TNFα or FasL as indicated. Cell lysates were subjected to immunoblotting, and NF-κB activation was monitored by the phosphorylation of IκBα. The same blot was reprobed using an Ab against total IκBα as internal control for protein loading.

(B) Wild-type or IκB mutant HT1080 cells were pretreated or not with MCD, DPPE, or DOPE, following stimulation with TNFα or FasL for 90 min. Viable cells were then washed and quantified 36 hr later, and the percentage of apoptotic cells was calculated.

Interfering with the Lipid Raft Composition Induces the Switch of TNFα-Mediated Signaling Pathway from NF-κB Activation to Apoptosis

Recruitment of TRADD, RIP, and TRAF2 to the TNFα signaling complex mediates IκBα kinase and subsequent NF-κB activation (Baldwin, 1996; Karin and Ben-Neriah, 2000). The NF-κB activity is regulated by inhibitor proteins (IκBs), which retain the transcription factor in the cytoplasm. Upon TNFα triggering, IκBs become phosphorylated by the IKK complex, resulting in their ubiquitylation and subsequent degradation by the proteasome, liberating NF-κB for translocation into the nucleus. In order to analyze NF-κB activation, we monitored the phosphorylation of IκBα. TNFα-induced phosphorylation of IκBα was observed 2 min after stimulation of HT1080 cells, whereas FasL stimulation had no effect (Figure 6A). We next investigated the role of lipid

rafts in TNFα-mediated NF-κB activation. As demonstrated in Figure 6A, disruption of lipid rafts by MCD-mediated cholesterol depletion completely abrogated TNFα-induced phosphorylation of IκBα. Similarly, DPPE that blocks activation-induced recruitment of signaling molecules to lipid rafts (Legler et al., 2001) also impaired IκBα phosphorylation (Figure 6A). In contrast, the nonraft lipid analog DOPE had no apparent effect on TNFα-mediated NF-κB activation. Under these conditions, both MCD and DPPE treatment did not affect the cell viability (see below). In control experiments (Figure 6A), TNFα-induced IκBα phosphorylation was not detected in IκB mutant HT1080 cells, which express a modified form of the NF-κB inhibitor IκBα that cannot be degraded due to mutated phosphorylation sites (Micheau et al., 2001).

Cell death induced by TNF family members is tightly regulated by genes that are activated by NF-κB, and modulation of the responses in favor of NF-κB protects cells from apoptosis. Stimulation with TNFα, contrary to FasL, did not induce apoptosis of HT1080 cells (Figure 6B). However, interfering with lipid rafts not only blocked TNFα-mediated NF-κB activation, but MCD- and DPPE-treated HT1080 cells also became susceptible to TNFα-mediated cell killing (Figure 6B). Disruption of lipid rafts by MCD provoked TNFα-induced apoptosis of 31% of wild-type HT1080 cells. Similarly, 28% of TNFα-stimulated cells died after saturation of lipid rafts by DPPE, whereas DOPE treatment had no effect. For comparison, 41% of IκB mutant HT1080 cells, which are unable to activate the NF-κB pathway and to synthesize antiapoptotic proteins such as FLIP, TRAFs, and IAPs (Micheau et al., 2001; Wang et al., 1998), underwent apoptosis when treated with TNFα (Figure 6B). On the other hand, FasL-mediated apoptosis of HT1080 cells was efficiently induced, irrespective of lipid raft integrity (Figure 6B).

Discussion

The crucial role of lipid rafts in signal transduction of several receptors, such as TCR, BCR, and FcγRs, has been well documented (Brown and London, 1998, 2000; Rietveld and Simons, 1998; Sedwick and Altman, 2002; Simons and Toomre, 2000). Whether signals induced by TNFR family members also depends on these specialized membrane microdomains has not been analyzed in detail or has revealed contradicting results, in particular concerning the membrane localization of TNFR1 (Cottin et al., 2002; Ko et al., 1999; Veldman et al., 2001) and Fas (Algeciras-Schimnich et al., 2002; Cremesti et al., 2001; Gajate and Mollinedo, 2001; Grassme et al., 2001a, 2001b; Hueber et al., 2002; Ko et al., 1999).

In the present study we therefore investigated the role of lipid rafts in signal transduction events of Fas and TNFR1, two members of the TNFR family expressed at the surface of the human fibrosarcoma cell line HT1080, which was found suitable for this study since it does not express TNFR2 as assessed by FACS as well as by functional analysis. Although a small amount of TNFR1 constitutively localized in microdomains of HT1080 cells, we found that, upon TNFα triggering, TNFR1 translocated to lipid rafts. This is in agreement with the localization of TNFR1 in nonactivated, transfected HeLa cells (Cottin et al., 2002) and the finding that TNFα stimulation resulted in a partial shift of TNFR1 to caveolin-enriched membrane microdomains in fibroblasts (Veldman et al., 2001). However, in the latter study, TNFR1 was not detected in lipid rafts of resting fibroblasts (Veldman et al., 2001), whereas in U937 and NIH-3T3 cells TNFR1 localized exclusively in caveolin-like domains even without TNFα triggering (Ko et al., 1999). Interestingly, the death domain of TNFR1 seems to be necessary and sufficient to target its receptor to lipid rafts (Cottin et al., 2002).

The functional importance of lipid rafts in TNFα-mediated signal transduction is still poorly understood. Culturing of U937 cells under lipoprotein-deficient conditions reduced the surface expression of both CD36 and TNFR1. Cholesterol depletion under these conditions blocked TNFα-induced apoptosis (Ko et al., 1999). Moreover, the study of sphingomyelinase activities associated with fibroblast caveolae revealed that TNFα stimulation activated a pool of the neutral sphingomyelinase localized outside of lipid rafts (Veldman et al., 2001). Here we demonstrate that the TNFα-induced signaling complex, consisting of TRADD, RIP, and TRAF2, was predominantly found in lipid rafts, whose integrity is required for the activation of the NF-κB pathway (Figure 3). In fact, disruption of lipid rafts by cholesterol depletion with MCD totally blocked TNFα-induced NF-κB activation by interfering with the recruitment of the IKK complex to the engaged TNFR1 (Figures 5 and 6). This result was confirmed when lipid rafts were saturated by exogenous insertion of DPPE into the cell membrane. DPPE partitions preferentially in sphingolipid/cholesterol-enriched microdomains and, unlike MCD, does not affect the integrity, the protein composition, and the ability to form clusters of lipid rafts, but inhibits activation-induced recruitment of signaling molecules to microdomains (Legler et al., 2001). Moreover, both types of reagents rendered a significant number of HT1080 cells susceptible to TNFα-induced apoptosis, in a manner comparable to cells expressing a modified repressor form of the NF-κB inhibitor IκBα (Figure 6). These findings are consistent with recently published observations showing that inhibition of NF-κB activation renders cells sensitive to TNFα-mediated killing by blocking NF-κB-dependant de novo synthesis of survival factors such as FLIP, TRAFs, and IAPs (Micheau et al., 2001; Wang et al., 1998). It is therefore tempting to speculate that, in raft-depleted/inactivated cells, a similar mechanism is responsible for the switch in the TNFα-induced signal transduction pathway from NF-κB activation toward cell death (Figure 7). It is well known that the TNFR1-TRADD-RIP-TRAF2 complex initiates the pathway leading to NF-κB activation, while the TNFR1-TRADD-FADD complex initiates the pathway leading to apoptosis (Hsu et al., 1996). However, we were not able to detect FADD in the TNFR1 signaling complex after MCD treatment (data not shown), indicating that additional mechanisms are responsible for modifying the outcome of TNFα signaling.

Contradictory results have also been published on the role of lipid rafts in Fas signaling. In one report, Fas was found to partition constitutively in lipid rafts of thymocytes and the B cell line SKW6.4 (Hueber et al., 2002). In other studies, only engaged Fas was shown to colo-

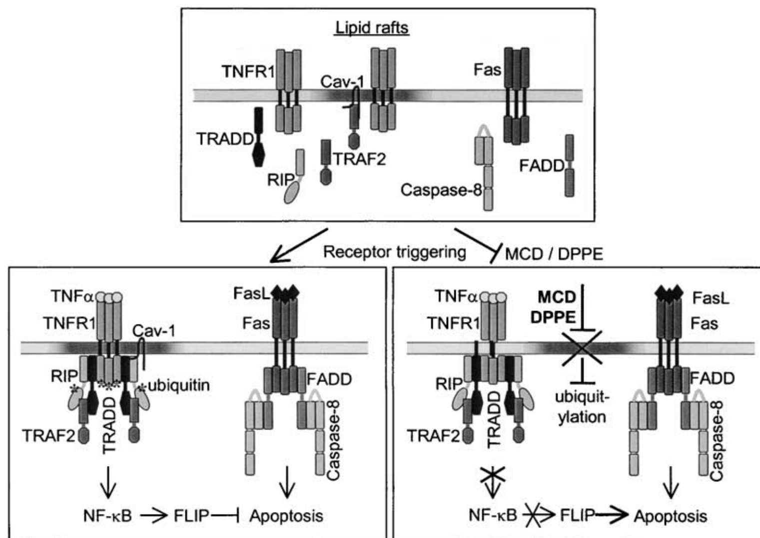

Figure 7. Lipid Rafts Are Essential for TNFα-Induced TNFR1 and RIP Ubiquitylation; Blocking Lipid Rafts Switches from NF-κB Activation to Apoptosis

In resting human HT1080 fibrosarcoma cells, TNFR1 partitions in lipid rafts, whereas Fas is excluded from these microdomains. Lipid rafts are enriched in cholesterol and caveolin-1. A fraction of TRAF2 localizes in lipid rafts due to protein-protein interaction with caveolin-1. Upon receptor engagement, TNFR1 translocates transiently to lipid rafts where it associates with TRADD, RIP, and TRAF2. Lipid rafts serve as platforms where the TNFα-mediated signaling complex accumulates to induce signal transduction events leading to NF-κB activation. NF-κB activation upregulates the expression of antiapoptotic factors, such as the caspase-8 inhibitor FLIP, which protects cells from apoptosis. Furthermore, lipid rafts are critical for TNFα-mediated TNFR1 and RIP ubiquitylation. Blocking lipid rafts by MCD or DPPE inhibits ubiquitylation of TNFR1 and RIP, blocks NF-κB activation, and may inhibit the synthesis of survival factors, such as FLIP, resulting in an increased sensitivity to TNFα-mediated apoptosis.

calize with the ganglioside GM1 in microdomains. In addition, Fas capping and FasL-induced apoptosis were both ceramide-dependent in Jurkat T cells and hepatocytes, thus suggesting the association of clustered Fas with microdomains (Cremesti et al., 2001; Grassme et al., 2001a, 2001b). This, however, is at odds with another study, which showed that lipid raft localization and capping of Fas in Jurkat and HL-60 cells was dependent on the presence of the ether lipid ET-18-OCH_3 (Gajate and Mollinedo, 2001). Yet another report showed that Fas clustering and FasL-induced apoptosis was independent of lipid rafts in Jurkat and SKW6.4 cells (Algeciras-Schimnich et al., 2002). Our results clearly demonstrate that FasL-induced signaling complex formation and apoptosis in HT1080 cells are independent of lipid rafts.

The lipid raft's localization of CD40, another member of the TNFR family, is less controversial (Hostager et al., 2000; Kaykas et al., 2001; Pham et al., 2002; Vidalain et al., 2000). Upon antibody or CD40L stimulation, TRAF2 and TRAF3 bind to CD40 in lipid rafts. The microdomain integrity is essential for CD40-mediated NF-κB activation since MCD-mediated cholesterol depletion was shown to block the translocation of TRAF molecules to lipid rafts and hampered their association with CD40 (Hostager et al., 2000; Vidalain et al., 2000). Moreover, TRAF2 and TRAF3 were degraded via the proteasome pathway after CD40 engagement (Brown et al., 2001), although the role of lipid rafts in this degradation process was not addressed.

The presence of modified forms of TNFR1-associated RIP in Triton X-100 cell lysates has been recently reported (Chen et al., 2002; Zhang et al., 2000). Our data confirm and extend these observations. In fact, using a method allowing the isolation of lipid rafts from nonionic detergent cell extracts, we provide direct evidence that, upon TNFα triggering, TNFR1 and RIP but not TRADD and TRAF2 are ubiquitylated in the cholesterol/sphingolipid-enriched membrane microdomains. Interfering with the structure and composition of membrane microdomains provided additional evidence for the crucial role of lipid rafts. MCD-mediated disruption of lipid rafts blocked the ubiquitylation of both TNFR1 and RIP, indicating that ubiquitylation is either required for translocation to microdomains or that ubiquitylation occurs within lipid rafts. To address this question, we specifically inhibited the activation-induced recruitment to lipid rafts without altering the protein composition of lipid rafts by exogenous addition of DPPE (Legler et al., 2001). DPPE, in contrast to DOPE, treatment of the cells impaired the modification of both TNFR1 and RIP, indicating that translocation precedes ubiquitylation and that the addition of ubiquitin takes place within microdomains (Figure 5). In addition, upon MCD or DPPE treatment, the amount of signaling molecules recruited to the engaged TNFR1 was reduced (RIP) or inhibited (IKKα and IKKβ), indicating that lipid rafts are also critical for the assembly and/or the stability of the TNFα-induced signaling complex.

Ubiquitylation can target proteins for degradation and may be critical for limiting inflammation by terminating TNFα-induced NF-κB activation. Lactacystin treatment resulted in an accumulation of modified protein providing evidence that ubiquitylation of TNFR1 and RIP leads to their degradation via the proteasome (Figure 4). We cannot, however, exclude that other modifications also take place in the signaling complex, but the changes in the apparent molecular weight observed on SDS-PAGE of TNFR1 and RIP cannot be explained exclusively by phosphorylation of the protein since phosphatase treatment did not decrease the amount of modified forms observed in the signaling complex (data not shown). Moreover, no sumoylation could be detected by Western blot analysis using sumo-specific Ab (data not

shown). In this context it is worthy to note that a protein related to the proteasomal subunit of the 26S proteasome, termed 55.11, was found to bind to the intracellular domain of TNFR1 (Boldin et al., 1995). Furthermore, the ubiquitin ligases Cbl and Nedd4 were shown to partition in lipid rafts upon IgE triggering (Lafont and Simons, 2001). Recently, it has also been shown that TRAF6 (which is not recruited to TNFR1) and TRAF2 function together with Ubc13/Uev1A to catalyze the ligation of unique polyubiquitin chains (Deng et al., 2000; Wang et al., 2001). To determine whether TRAF2 may act as an E3 ubiquitin ligase in the ubiquitylation of TNFR1 and RIP, we transfected HT1080 cells with a modified TRAF2 whose RING finger domain was deleted (Devin et al., 2000; Rothe et al., 1995). Overexpression of this modified TRAF2 had no apparent effect on the ubiquitylation of TNFR1 and RIP (data not shown), indicating that TRAF2 is probably not the ubiquitin ligase responsible for the modification of TNFR1 and RIP. To gain further information on ubiquitylation, we overexpressed various myc-tagged RIP mutants in HT1080 cells. Following stimulation and immunoprecipitation of engaged TNFR1 signaling complex, no significant change of TNFR1 and RIP modification could be observed by Western blot analysis using anti-myc, anti-TNFR1, or anti-RIP antibodies in cells overexpressing the K45R mutant and a mutant lacking the kinase domain (ΔKD) (Holler et al., 2000; Ting et al., 1996), indicating that, at least for RIP, more than one lysine is ubiquitylated upon TNFα stimulation. In HT1080 cells overexpressing a mutant lacking the death domain (ΔDD), no significant alteration of the modification of endogenous TNFR1 and RIP was observed, whereas the recruitment of RIP(ΔDD) to engaged TNFR1 was impaired. Further investigations such as site-directed mutations of the potential ubiquitylated lysine residues or ubiquitylation regulatory regions of the proteins are necessary to gain more information about the functional significance of this structural processing of both proteins.

In conclusion, we demonstrate that lipid rafts serve as platforms for TNFα-mediated signaling by recruiting adaptor molecules, such as TRADD and TRAF2, and kinases, like RIP and the IKK complex, to microdomains leading to NF-κB activation. In addition, these microdomains play a critical role in the specific modification of TNFR1 and RIP by ubiquitin. Disruption of lipid rafts by cholesterol depletion or blocking activation-induced recruitment of signaling molecules to lipid rafts by DPPE inhibits the triggering of the NF-κB pathway and therefore induces the switch of TNFα-mediated responses toward apoptosis.

Experimental Procedures

Cells

Human fibrosarcoma cells HT1080, wild-type and IκB mutant (Micheau et al., 2001), were cultured in high glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 5 μg/ml penicillin, 5 μg/ml streptomycin, and 10 μg/ml neomycin (GIBCO-BRL Life Technologies Inc., Paisley, United Kingdom) and grown in 5% CO_2 at 37°C. HT1080 cells were stably transfected with wild-type TRAF2 and TRAF2 lacking the RING finger domain ($TRAF2_{87-501}$ [Devin et al., 2000], kindly provided by H. Wajant), or mutants of RIP (K45R, ΔKD, ΔDD [Ting et al., 1996]). Alternatively, cells were transiently tranfected using the calcium phosphate method with a HA-tagged ubiquitin (Treier et al., 1994) (kindly provided by W. Krek).

Antibodies

Rabbit anti-EGFR pAb was from Upstate Biotechnology (Lake Placid, NY). Rabbit anti-Fas pAb C20, mouse anti-TNFR1 mAb H-5, rabbit anti-TRAF2 pAb C20, rabbit anti-Fyn pAb FYN3, and rabbit anti-HA pAb were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit anti-phospho-IκBα pAb and rabbit anti-total-IκBα pAb were from New England BioLabs (Allschwil, Switzerland). Rabbit anti-caveolin-1 pAb, mouse anti-FADD mAb, mouse anti-TRADD mAb, mouse anti-IKKβ mAb, and mouse anti-RIP mAb were from Transduction Laboratories (Lexington, KY). Mouse anti-caspase-8 mAb was from MBL (Nunningen, Switzerland). Mouse anti-ubiquitin mAb FK2 was from Affiniti Research Products Ltd (Exeter, United Kingdom). Mouse anti-IKKα mAb was from Pharmingen (BD, Erembodegem, Belgium). CTx^{HRP} was from Sigma (Buchs, Switzerland). HRP-conjugated goat ant-mouse IgG1, IgG2a, and IgG2b Ab were from Southern Biotechnology Associates (Birmingham, AL). HRP-conjugated goat anti-rabbit IgG Ab was from Jackson ImmunoResearch (West Grove, PA).

Ligands, Lipids, and Chemicals

Human recombinant Flag-tagged TNFα and Flag-tagged Mega-FasL were from Apotech (Epalinges, Switzerland). Dipalmitoyl-phosphatidylethanolamine (DPPE) and dioleoyl-phosphatidylethanolamine (DOPE) were purchased from Sigma, and liposomes at 2 mg/ml were generated in PBS containing 30 mM β-octyl-glucopyranoside (Fluka, Buchs, Switzerland) and used at 50 μg/ml in serum-free medium as described (Legler et al., 2001). Methyl-β-cyclodextrin (Sigma) was used at 25 mM final concentration. To block proteasome-mediated protein degradation, cells were incubated in medium containing 40 μM lactacystin (Affiniti Research Products Ltd) for 30 min at 37°C prior to stimulation as described (Shen et al., 1997). Cells were labeled with ^{14}C cholesterol as described previously (Legler et al., 2001), and the cholesterol distribution was analyzed in Triton X-100 soluble and insoluble fractions.

Lipid Raft Isolation and Western Blotting

Lipid rafts were isolated by sucrose density gradient centrifugation essentially as described (Zhang et al., 1998). In brief, subconfluent HT1080 cells from two 15 cm culture dishes were lysed on ice for 20 min in 2 ml of MNX buffer (1% Triton X-100 in 25 mM MES, 150 mM NaCl [pH 6.5]) supplemented with 10 μg/ml benzamidine, 2 μg/ml antipain, and 1 μg/ml leupeptin, and homogenized (10 strokes) with a loose-fitting glass dounce homogenizer (Polylabo, Illkirch, France). The homogenates were mixed with 2 ml 90% sucrose made with MN buffer and placed on the bottom of a centrifuge tube. The samples were then overlaid with 4 ml of 35% sucrose and 4 ml of 5% sucrose and centrifugated at 175,000 g in a Centrikon T-2070 centrifuge (Kontron Analytics, Zürich, Switzerland) for 16 hr at 4°C. One milliliter fractions were collected from the top of the gradient and analyzed by Western blotting. The pellet present at the bottom of the gradient was sonicated in 1 ml of MNX.

Alternatively, Triton X-100 insoluble raft fractions were prepared essentially as described (Legler et al., 2001). In brief, cells (2×10^6) were lysed on ice for 20 min in 200 μl of MNX buffer. The cell lysate was homogenized as above and spun at 500 g for 7 min at 4°C; the nuclear pellet was washed sequentially in MNX and 0.5 % Brij 78 in PBS. The postnuclear supernatant was centrifuged at 100,000 g for 1 hr at 4°C. The lipid raft fraction in the pellet was resuspended in 200 μl of 0.5 % Brij 78; nonsoluble material was removed by an additional centrifugation. The 100,000 g supernatant is referred to as the Triton X-100 soluble fraction containing the phospholipid membrane and cytosolic fraction.

Proteins from total cell lysates and immunoprecipitates were resolved by SDS-PAGE and transferred to Protran nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). Nonspecific binding sites were blocked by incubation in PBS containing 0.05% Tween 20 and 5% dry milk. Immunoblots were then incubated with specific primary Ab followed by HRP-conjugated secondary Ab and were developed by the enhanced chemiluminescence method according to the manufacturer's protocol (Pierce, Rockford, IL).

Receptor-Induced Signaling Complex Analysis

A total of 10^8 HT1080 cells in 1 ml of medium were stimulated with 2 μg/ml of TNFα or FasL for the indicated times at 37°C, washed

with cold PBS, lysed in 4 ml of MNX buffer, and subjected to lipid raft isolation. To immunoprecipitate the total amount of receptor, 5×10^7 cells were lysed in MNX, the ligand was added, and the lysate was fractionated. Soluble and lipid raft fractions were pre-cleared, and the signaling complex was immunoprecipitated overnight at 4°C with anti-Flag M2-agarose beads (Sigma) Alternatively, ubiquitylated proteins of Triton X-100 soluble and insoluble raft fractions were immunoprecipitated using an anti-ubiquitin mAb and protein G Sepharose beads. Beads were washed four times with 1% Triton X-100 or 0.5% Brij 78, respectively. Where indicated, the TNFα signaling complex was dissociated in 50 μl of PBS containing 1% SDS by boiling for 10 min and diluted 20-fold with lysis buffer before it was subjected to a second immunoprecipitation using 10 μg/ml of an anti-ubiquitin Ab.

NF-κB Activation

Cells were treated or not with MCD, DPPE, or DOPE for 30 min, washed, and stimulated with 0.2 μg/ml TNFα or FasL for 2 min. Cells were immediately washed with cold PBS, lysed, and subjected to Western blot analysis to detect phosphorylated IκBα.

Cell Death and Viability Assay

Cells were treated or not with MCD, DPPE, or DOPE and stimulated for 90 min with 10 μg/ml of TNFα, 1 μg/ml of FasL, or anti-TNFR2 (UTR1) Ab. Cells were extensively washed, seeded at 1.5×10^4 cells/well in 96-well microtiter plates, and cultured for 36 hr, and viable cells were stained with methylene blue and quantified by measuring the optical density at 630 nm.

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References

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

Aravind, L., Dixit, V.M., and Koonin, E.V. (2001). Apoptotic molecular machinery: vastly increased complexity in vertebrates revealed by genome comparisons. Science *291*, 1279–1284.

Ashkenazi, A., and Dixit, V.M. (1998). Death receptors: signaling and modulation. Science *281*, 1305–1308.

Baker, S.J., and Reddy, E.P. (1998). Modulation of life and death by the TNF receptor superfamily. Oncogene *17*, 3261–3270.

Baldwin, A.S., Jr. (1996). The NF-κB and IκB proteins: new discoveries and insights. Annu. Rev. Immunol. *14*, 649–683.

Balkwill, F., Foxwell, B., and Brennan, F. (2000). TNF is here to stay! Immunol. Today *21*, 470–471.

Baud, V., and Karin, M. (2001). Signal transduction by tumor necrosis factor and its relatives. Trends Cell Biol. *11*, 372–377.

Boldin, M.P., Mett, I.L., and Wallach, D. (1995). A protein related to a proteasomal subunit binds to the intracellular domain of the p55 TNF receptor upstream to its 'death domain'. FEBS Lett. *367*, 39–44.

Brown, D.A., and London, E. (1998). Structure and origin of ordered lipid domains in biological membranes. J. Membr. Biol. *164*, 103–114.

Brown, D.A., and London, E. (2000). Structure and function of sphingolipid- and cholesterol-rich membrane rafts. J. Biol. Chem. *275*, 17221–17224.

Brown, K.D., Hostager, B.S., and Bishop, G.A. (2001). Differential signaling and tumor necrosis factor receptor-associated factor (TRAF) degradation mediated by CD40 and the Epstein-Barr virus oncoprotein latent membrane protein 1 (LMP1). J. Exp. Med. *193*, 943–954.

Budd, R.C. (2001). Activation-induced cell death. Curr. Opin. Immunol. *13*, 356–362.

Chen, G., Cao, P., and Goeddel, D.V. (2002). TNF-induced recruitment and activation of the IKK complex require Cdc37 and Hsp90. Mol. Cell *9*, 401–410.

Cottin, V., Doan, J.E., and Riches, D.W. (2002). Restricted localization of the TNF receptor CD120a to lipid rafts: a novel role for the death domain. J. Immunol. *168*, 4095–4102.

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

Deng, L., Wang, C., Spencer, E., Yang, L., Braun, A., You, J., Slaughter, C., Pickart, C., and Chen, Z.J. (2000). Activation of the IκB kinase complex by TRAF6 requires a dimeric ubiquitin-conjugating enzyme complex and a unique polyubiquitin chain. Cell *103*, 351–361.

Devin, A., Cook, A., Lin, Y., Rodriguez, Y., Kelliher, M., and Liu, Z. (2000). The distinct roles of TRAF2 and RIP in IKK activation by TNF-R1: TRAF2 recruits IKK to TNFR1 while RIP mediates IKK activation. Immunity *12*, 419–429.

Dhein, J., Daniel, P.T., Trauth, B.C., Oehm, A., Moller, P., and Krammer, P.H. (1992). Induction of apoptosis by monoclonal antibody anti-APO-1 class switch variants is dependent on cross-linking of APO-1 cell surface antigens. J. Immunol. *149*, 3166–3173.

Feng, X., Gaeta, M.L., Madge, L.A., Yang, J.H., Bradley, J.R., and Pober, J.S. (2001). Caveolin-1 associates with TRAF2 to form a complex that is recruited to tumor necrosis factor receptors. J. Biol. Chem. *276*, 8341–8349.

Gajate, C., and Mollinedo, F. (2001). The antitumor ether lipid ET-18-OCH(3) induces apoptosis through translocation and capping of Fas/CD95 into membrane rafts in human leukemic cells. Blood *98*, 3860–3863.

Grassme, H., Jekle, A., Riehle, A., Schwarz, H., Berger, J., Sandhoff, K., Kolesnick, R., and Gulbins, E. (2001a). CD95 signaling via ceramide-rich membrane rafts. J. Biol. Chem. *276*, 20589–20596.

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

Harder, T., Scheiffele, P., Verkade, P., and Simons, K. (1998). Lipid domain structure of the plasma membrane revealed by patching of membrane components. J. Cell Biol. *141*, 929–942.

Holler, N., Zaru, R., Micheau, O., Thome, M., Attinger, A., Valitutti, S., Bodmer, J.L., Schneider, P., Seed, B., and Tschopp, J. (2000). Fas triggers an alternative, caspase-8-independent cell death pathway using the kinase RIP as effector molecule. Nat. Immunol. *1*, 489–495.

Hostager, B.S., Catlett, I.M., and Bishop, G.A. (2000). Recruitment of CD40 and tumor necrosis factor receptor-associated factors 2 and 3 to membrane microdomains during CD40 signaling. J. Biol. Chem. *275*, 15392–15398.

Hsu, H., Xiong, J., and Goeddel, D.V. (1995). The TNF receptor 1-associated protein TRADD signals cell death and NF-κB activation. Cell *81*, 495–504.

Hsu, H., Shu, H.B., Pan, M.G., and Goeddel, D.V. (1996). TRADD-TRAF2 and TRADD-FADD interactions define two distinct TNF receptor 1 signal transduction pathways. Cell *84*, 299–308.

Hueber, A.O., Bernard, A.M., Herincs, Z., Couzinet, A., and He, H.T. (2002). An essential role for membrane rafts in the initiation of Fas/CD95-triggered cell death in mouse thymocytes. EMBO Rep. *3*, 190–196.

Janes, P.W., Ley, S.C., and Magee, A.I. (1999). Aggregation of lipid rafts accompanies signaling via the T cell antigen receptor. J. Cell Biol. *147*, 447–461.

Karin, M., and Ben-Neriah, Y. (2000). Phosphorylation meets ubiqui-

tination: the control of NF-κB activity. Annu. Rev. Immunol. *18*, 621–663.

Kaykas, A., Worringer, K., and Sugden, B. (2001). CD40 and LMP-1 both signal from lipid rafts but LMP-1 assembles a distinct, more efficient signaling complex. EMBO J. *20*, 2641–2654.

Kelliher, M.A., Grimm, S., Ishida, Y., Kuo, F., Stanger, B.Z., and Leder, P. (1998). The death domain kinase RIP mediates the TNF-induced NF-κB signal. Immunity *8*, 297–303.

Ko, Y.G., Lee, J.S., Kang, Y.S., Ahn, J.H., and Seo, J.S. (1999). TNFα-mediated apoptosis is initiated in caveolae-like domains. J. Immunol. *162*, 7217–7223.

Krammer, P.H. (2000). CD95's deadly mission in the immune system. Nature *407*, 789–795.

Lafont, F., and Simons, K. (2001). Raft-partitioning of the ubiquitin ligases Cbl and Nedd4 upon IgE-triggered cell signaling. Proc. Natl. Acad. Sci. USA *98*, 3180–3184.

Legler, D.F., Doucey, M.A., Cerottini, J.C., Bron, C., and Luescher, I.F. (2001). Selective inhibition of CTL activation by a dipalmitoyl-phospholipid that prevents the recruitment of signaling molecules to lipid rafts. FASEB J. *15*, 1601–1603.

Liu, Z.G., Hsu, H., Goeddel, D.V., and Karin, M. (1996). Dissection of TNF receptor 1 effector functions: JNK activation is not linked to apoptosis while NF-κB activation prevents cell death. Cell *87*, 565–576.

Locksley, R.M., Killeen, N., and Lenardo, M.J. (2001). The TNF and TNF receptor superfamilies: integrating mammalian biology. Cell *104*, 487–501.

Micheau, O., Lens, S., Gaide, O., Alevizopoulos, K., and Tschopp, J. (2001). NF-κB signals induce the expression of c-FLIP. Mol. Cell. Biol. *21*, 5299–5305.

Muller, S., Hoege, C., Pyrowolakis, G., and Jentsch, S. (2001). SUMO, ubiquitin's mysterious cousin. Nat. Rev. Mol. Cell Biol. *2*, 202–210.

Natoli, G., Costanzo, A., Guido, F., Moretti, F., and Levrero, M. (1998). Apoptotic, non-apoptotic, and anti-apoptotic pathways of tumor necrosis factor signaling. Biochem. Pharmacol. *56*, 915–920.

Pham, L.V., Tamayo, A.T., Yoshimura, L.C., Lo, P., Terry, N., Reid, P.S., and Ford, R.J. (2002). A CD40 signalosome anchored in lipid rafts leads to constitutive activation of NF-κB and autonomous cell growth in B cell lymphomas. Immunity *16*, 37–50.

Rietveld, A., and Simons, K. (1998). The differential miscibility of lipids as the basis for the formation of functional membrane rafts. Biochim. Biophys. Acta *1376*, 467–479.

Rothe, M., Sarma, V., Dixit, V.M., and Goeddel, D.V. (1995). TRAF2-mediated activation of NF-κB by TNF receptor 2 and CD40. Science *269*, 1424–1427.

Sedwick, C.E., and Altman, A. (2002). Ordered just so: lipid rafts and lymphocyte function. Science's STKE, http://stke.sciencemag.org/cgi/content/full/sigtrans;2002/122/re2.

Shen, Z., Reznikoff, G., Dranoff, G., and Rock, K.L. (1997). Cloned dendritic cells can present exogenous antigens on both MHC class I and class II molecules. J. Immunol. *158*, 2723–2730.

Simons, K., and Toomre, D. (2000). Lipid rafts and signal transduction. Nat. Rev. Mol. Cell Biol. *1*, 31–39.

Ting, A.T., Pimentel-Muinos, F.X., and Seed, B. (1996). RIP mediates tumor necrosis factor receptor 1 activation of NF-κB but not Fas/APO-1-initiated apoptosis. EMBO J. *15*, 6189–6196.

Treier, M., Staszewski, L.M., and Bohmann, D. (1994). Ubiquitin-dependent c-Jun degradation in vivo is mediated by the Δ domain. Cell *78*, 787–798.

Veldman, R.J., Maestre, N., Aduib, O.M., Medin, J.A., Salvayre, R., and Levade, T. (2001). A neutral sphingomyelinase resides in sphingolipid-enriched microdomains and is inhibited by the caveolin-scaffolding domain: potential implications in tumour necrosis factor signalling. Biochem. J. *355*, 859–868.

Vidalain, P.O., Azocar, O., Servet-Delprat, C., Rabourdin-Combe, C., Gerlier, D., and Manie, S. (2000). CD40 signaling in human dendritic cells is initiated within membrane rafts. EMBO J. *19*, 3304–3313.

Wallach, D., Varfolomeev, E.E., Malinin, N.L., Goltsev, Y.V., Kovalenko, A.V., and Boldin, M.P. (1999). Tumor necrosis factor receptor and Fas signaling mechanisms. Annu. Rev. Immunol. *17*, 331–367.

Wang, C.Y., Mayo, M.W., Korneluk, R.G., Goeddel, D.V., and Baldwin, A.S., Jr. (1998). NF-κB antiapoptosis: induction of TRAF1 and TRAF2 and c-IAP1 and c-IAP2 to suppress caspase-8 activation. Science *281*, 1680–1683.

Wang, C., Deng, L., Hong, M., Akkaraju, G.R., Inoue, J., and Chen, Z.J. (2001). TAK1 is a ubiquitin-dependent kinase of MKK and IKK. Nature *412*, 346–351.

Zhang, W., Trible, R.P., and Samelson, L.E. (1998). LAT palmitoylation: its essential role in membrane microdomain targeting and tyrosine phosphorylation during T cell activation. Immunity *9*, 239–246.

Zhang, S.Q., Kovalenko, A., Cantarella, G., and Wallach, D. (2000). Recruitment of the IKK signalosome to the p55 TNF receptor: RIP and A20 bind to NEMO (IKKγ) upon receptor stimulation. Immunity *12*, 301–311.