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

Daniel F. Legler,1,2,* Olivier Micheau,1 Marie-Agnès Doucey,1 Jürg Tschopp,1 and Claude Bron1
1Institute of Biochemistry
University of Lausanne
BIL Biomedical Research Center
CH-1012 Epalinges
Switzerland
2Division of Immunology
Department of Biology
University of Konstanz
Universitätsstrasse 10
D-78457 Konstanz
Germany

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; Budd, 2001; Krammer, 2000). The DD of Fas (CD95) interacts with the DD of the death domain protein (FADD/MORT1) to induce the signaling pathway leading to cell death (Hsu et al., 1996; Krammer, 2000). The DD 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). Binding of Fasl induces receptor activation resulting 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 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-

*Correspondence: daniel.legler@uni-konstanz.de

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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 IL-1 receptor (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).

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 CtxHRP.

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 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 pro-caspase-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).
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, IκBα, IκBβ, FADD, caspase-8, and caveolin-1.

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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 immunoblotted using specific Ab.
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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 ^14C 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 treated or not with MCD, stimulus 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 14C cholesterol and treated or not with MCD, and the distribution of 14C 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 with MCD, DPPE, or DOPE, and the distribution of 14C cholesterol was measured in Triton X-100 soluble and insoluble lipid raft fractions.

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. In contrast, the non-raft 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 IκBα mutant HT1080 cells, which express a modified form of the NF-κB inhibitor IκBα that cannot be degraded due to the NF-κB inhibitor IκBα that cannot be degraded due to 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 TNFRII 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-
on the presence of the ether lipid ET-18-OCH3 (Gajate lipid rafts. To address this question, we specifically study, which showed that lipid raft localization and capping that ubiquitylation is either required for translocation of the protein since phosphatase treat-

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-OCH3, (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 (Alge-ciras-Schimmich 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/sphingo-lipid-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 transloca-tion to microdomains or that ubiquitylation occurs within lipid rafts. To address this question, we specifically in-hibited 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 treat-

ment 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-TNFRI, 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 ΔB mutant (Michaël 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% CO2 at 37°C. HT1080 cells were stably transfected with wild-type TRAF2 and TRAF2 lacking the RING finger domain (TRAF2ΔR) (Devin et al., 2000, kindly provided by W. Wajntant), or mutants of RIP (K45R, ΔKD, ΔDD [Ting et al., 1996]). Alternatively, cells were transiently transfected 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-TNFRI 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-κB pAb and rabbit anti-total-κB pAb were from New England Biolabs (Alachniville, Switzerland). Rabbit anti-caveolin-1 pAb, mouse anti-FADD mAb, mouse anti-IκKα pAb, and mouse anti-RIP mAb were from Transduction Laboratories (Lexington, KY). Mouse anti-caspase-8 mAb was from MBL (Nuningen, Switzerland). Mouse anti-ubiquitin mAb FK2 was from Affiniti Research Products Ltd (Exeter, United Kingdom). Mouse anti-IκKβ mAb was from Pharmingen (BD, Embredgegen, Belgium). Ctxαβ2 was from Sigma (Buchs, Switzerland). HRP-conjugated goat anti-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 Apetech (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 proteosome-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 13C 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 MNX 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 centrifuged at 175,000 g in a Centrifuge 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 × 106) 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 factor.

**Receptor-Induced Signaling Complex Analysis**

A total of 10^6 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 x 10^7 cells were lysed in MNX, the ligand was added, and the lysate was fractionated. Soluble and lipid raft fractions were precleared, and the signaling complex was immunoprecipitated overnight at 4°C with anti-Flag M2-agarose beads (Sigma) Alternatively, oncoprotein latent membrane protein 1 (LMP1). J. Exp. Med.

Cleared, and the signaling complex was immunoprecipitated over-night at 4°C with anti-Flag M2-agarose beads (Sigma) Alternatively, oncoprotein latent membrane protein 1 (LMP1). J. Exp. Med.

Soluble and lipid 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 IkBα.

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-TNFα (UTRI) Ab. Cells were extensively washed, seeded at 1.5 x 10^5 cells/well in 96-well microtiter plates, and cultivated 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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