CD95 Stimulation Results in the Formation of a Novel Death Effector Domain Protein-containing Complex

Received for publication, January 31, 2008, and in revised form, July 8, 2008. Published, JBC Papers in Press, July 17, 2008, DOI 10.1074/jbc.M800823200

Inna N. Lavrik, Thomas Mock, Alexander Golks 1, Julia C. Hoffmann, Simone Baumann, and Peter H. Krammer 2

From the Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany

Stimulation of CD95 (APO-1/Fas) by its natural ligand CD95L (APo-1L/FasL) leads to the formation of the death-inducing signaling complex. Here we report that upon CD95 stimulation in several T and B cell lines, a novel signaling complex is formed, which we term complex II. Complex II is composed of the death effector domain proteins as follows: procaspase-8a/b, three isoforms of c-FLIP (c-FLIP L, c-FLIP S, c-FLIP R), and FADD. Notably, complex II does not contain CD95. Based on our findings we suggest that CD95 signaling includes two steps. The first step involves formation of the death-inducing signaling complex at the cell membrane. The second step involves formation of the cytosolic death effector domain protein-containing complex that may play an important role in amplification of caspase activation.

Apoptotic cell death is common in multicellular organisms. Apoptosis can be triggered by a number of factors, including UV- or γ-irradiation, chemotherapeutic drugs, and signaling from death receptor (1). CD95 (APO-1/Fas) is a member of the death receptor family, a subfamily of the tumor necrosis factor receptor superfamily (1–3). Cross-linking of CD95 with its natural ligand CD95L (CD178) (4) or with agonistic antibodies such as anti-APO-1 (5) induces apoptosis in sensitive cells.

Two CD95 signaling pathways were established (12). Type I cells are characterized by high levels of CD95 DISC formation and increased amounts of active caspase-8. Activated caspase-8 directly leads to the activation of downstream effector caspases-3 and -7. Type II cells are characterized by lower levels of CD95 DISC formation and thus lower levels of active caspase-8. In this case, signaling requires an additional amplification loop that involves the cleavage by caspase-8 of the Bcl-2 family protein Bid to generate truncated Bid and subsequent truncated Bid-mediated release of cytochrome c from mitochondria (10). The release of cytochrome c from mitochondria results in apoptosis formation followed by activation of caspase-9, which in turn cleaves downstream effector caspases.

In this study we reveal the formation of a novel cytosolic signaling complex, which occurs upon CD95 stimulation. This complex includes only the DED-containing proteins procaspase-8, c-FLIP, and FADD and does not contain CD95. This complex plays an important role in the amplification of caspase activation.

EXPERIMENTAL PROCEDURES

Cell Lines—The B lymphoblastoid cell lines SKW6.4, Raji, and BJAB and the T cell lines HUT78, Jurkat clones 16, and A3 were maintained in RPMI 1640 medium (Invitrogen), 10 mM HEPES (Invitrogen), 50 μg/ml gentamycin (Invitrogen), and 10% fetal calf serum (Invitrogen) in 5% CO 2.

Antibodies and Reagents—Anti-CD95 polyclonal antibodies (C20), anti-TRAF2 monoclonal antibodies (IgG1), and anti-caspase-9 monoclonal antibodies (IgG2a) were purchased from Santa Cruz Biotechnology. Anti-ERK1 monoclonal antibodies (mouse IgG1), anti-RIP1 monoclonal antibodies (IgG2a), and anti-PARP monoclonal antibodies (mouse IgG1) were from BD Transduction Laboratories. Anti-caspase-3 polyclonal antibodies were from Cell Signaling (Frankfurt am Main, Germany). The anti-FADD monoclonal antibodies 1C4 (mouse IgG1) recognize the C terminus of FADD (13). The anti-caspase-8 monoclonal antibodies C15 (mouse IgG2b) recognize the p18 subunit of caspase-8 (14). Anti-FLIP monoclonal antibodies NF6 (mouse IgG1) recognize the N terminus of FLIP.

Abbreviations used are: DISC, death-inducing signaling complex; DED, death effector domain; C8, caspase-8; IP, immunoprecipitation; WB, Western blot; BID, Bcl-2/BNIP3-like protein; APO-1, apoptosis inducing protein-1; FADD, death activating domain-containing protein; FLIP, FADD-like interleukin 1 β-converting enzyme (ICE)-like protein; TRAF, tumor necrosis factor receptor-associated factor; PARP, poly(ADP-ribos)e polymerase; LZ, leucine zipper; ERK, extracellular signal-regulated kinase.

This work was supported by Grant SB 405 from Wilhelm Sander Stiftung, SB Cancer and Tumorzentrum, Heidelberg/Mannheim, Germany. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 Present address: Novartis Pharma AG, CH-4002 Basel, Switzerland.

2 To whom correspondence should be addressed: Division of Immunogenetics, Tumor Immunology Program, German Cancer Research Center, Im Neuenheimer Feld 280, 69120 Heidelberg, Germany. E-mail: p.krammer@dkfz.de.

These abbreviations are used: DISC, death-inducing signaling complex; DED, death effector domain; C8, caspase-8; IP, immunoprecipitation; WB, Western blot; BID, Bcl-2/BNIP3-like protein; APO-1, apoptosis inducing protein-1; FADD, death activating domain-containing protein; FLIP, FADD-like interleukin 1 β-converting enzyme (ICE)-like protein; TRAF, tumor necrosis factor receptor-associated factor; PARP, poly(ADP-ribos)e polymerase; LZ, leucine zipper; ERK, extracellular signal-regulated kinase.
Anti-APO-1 is an agonistic monoclonal antibody recognizing an epitope on the extracellular part of CD95 (APO-1/Fas) (5). LZ-CD95L was prepared as described (15). Horseradish peroxidase-conjugated goat anti-mouse IgG1 and -2b were from Southern Biotechnology Associates (Birmingham, AL). Z-IETD-AFC and Z-DEVD-AFC were from Molecular Probes (Leiden, Netherlands). All other chemicals used were of analytical grade and purchased from Merck or Sigma.

### Caspase Activity Assays

Cytosolic lysates were incubated with 50 μM site-specific tetrapeptide substrates (Z-IETD-AFC for caspase-8 and Z-DEVD-AFC for caspase-3) in a caspase assay buffer B (50 mM HEPES, 100 mM NaCl, 10 mM dithiothreitol, 0.1% (w/v) CHAPS, 10% (w/v) sucrose, pH 7.4) in a final volume of 200 μl. The release of the fluorogenic group AFC was determined after 1 h of incubation at 37 °C by a microplate fluorescence reader Wallach 1420 (PerkinElmer Life Sciences) at the excitation wavelength of 405 nm and emission wavelength of 535 nm.

### DISC Analysis by Immunoprecipitation and Western Blot

1 x 10⁶ cells were treated with the indicated amounts of LZ-CD95L at 37 °C for the indicated times, washed twice in 1× PBS, and subsequently lysed in buffer A (20 mM Tris/HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (Sigma), protease inhibitor mixture (Roche Applied Science), 1% Triton X-100 (Serva, Heidelberg, Germany), and 10% glycerol) or lysed without treatment. The CD95 DISC was immunoprecipitated overnight with 2 μg of anti-APO-1 and protein A-Sepharose beads. Protein A-Sepharose beads were washed five times with 20 volumes of lysis buffer. The immunoprecipitates were analyzed on 12% polyacrylamide gel. Subsequently, the gels were transferred to Hybond nitrocellulose membrane (Amer sham Biosciences), blocked with 5% nonfat dry milk in PBS/Tween (PBS plus 0.05% Tween 20) for 1 h, washed with PBS/Tween, and incubated with the primary antibodies in PBS/Tween at 4 °C overnight. Blots were developed with a chemiluminescence method following the manufacturer’s protocol (PerkinElmer Life Sciences).

### Caspase-8 and FADD Immunoprecipitations

1 x 10⁶ cells were treated with LZ-CD95L at 37 °C for the indicated times, washed twice in 1× PBS, and subsequently lysed in buffer A. Caspase-8 and FADD immunoprecipitations were carried out using anti-caspase-8 antibodies C15 and anti-FADD antibodies 1C4, respectively. Immunoprecipitations were performed and analyzed as described for the DISC analysis. Anti-caspase-8

---

**Figure 1. Caspase-8 activation upon stimulation with different amounts of LZ-CD95L.** B lymphoblastoid SKW6.4 cells were stimulated with different concentrations of LZ-CD95L for indicated time intervals. A, specific cell death was measured 18 h after stimulation. B, cleavage of procaspases-8 and -3 as well as PARP cleavage was analyzed by Western blot (WB). Anti-ERK Western blot was used as a loading control. IETD-AFC activity was measured upon stimulation with 1 μg/ml LZ-CD95L (C) or 100 ng/ml LZ-CD95L (E). DEVD-AFC activity was measured upon stimulation with 1 μg/ml of LZ-CD95L (D) or 100 ng/ml of LZ-CD95L (F).
antibodies are IgG2b; however, IgGs were not always detected via Western blot probably due to modification of the IgG2b epitope during PAGE. Anti-FADDs are monoclonal IgG1 antibodies. The Western blot analysis of FADD immunoprecipitations with the secondary IgG1 antibodies typically resulted in the detection of IgGs.

Flow Cytometry Analysis—The percentage of viable cells was determined by FSC/SSC using a FACSscan cytometer (BD Biosciences). A minimum of 10,000 cells per sample was analyzed. Specific cell death was calculated as follows: (percentage of experimental cell death − percentage of spontaneous cell death) / (100 − percentage of spontaneous cell death) × 100.

In Vitro Translation and in Vitro Caspase-3 Cleavage Assays—Complex II was immunoprecipitated from 1 × 10⁸ SKW6.4 cells. To accomplish this task, 1 × 10⁸ cells were treated with LZ-CD95L at 37 °C for the indicated times, washed twice in 1× PBS, and subsequently lysed in buffer A. Afterward the lysates were immunodepleted from the DISC with two steps of DISC immunoprecipitation with 2 µg of anti-APO-1 and 30 µl of protein A-Sepharose beads. Then the supernatants were depleted from anti-APO-1 antibodies with three steps of incubation with 30 µl of protein A beads. Finally, complex II was immunoprecipitated from the remaining supernatant with C15 antibodies. Immunoprecipitates were incubated with in vitro translated ³⁵S-labeled procaspase-3 (TnT, T7 coupled reticulocyte lysate system, Promega) in caspase cleavage buffer (50 mM HEPES, pH 7.4, 100 mM NaCl, 0.1% CHAPS, 10 mM dithiothreitol, and 10% sucrose) at 4 °C overnight. Cleavage reactions were separated on 12% polyacrylamide gel, blotted, and subjected to Western blot analysis.

RESULTS

CD95 Stimulation with Different Concentrations of LZ-CD95L

Results in Different Kinetics of Caspase-8 Activation—B lymphoblastoid SKW6.4 cells were stimulated with different amounts of LZ-CD95L to analyze kinetics of caspase activation. Stimulation of various cells with LZ-CD95L was shown to activate the CD95-mediated apoptotic pathway: CD95 DISC formation, caspase-8 activation, and thereby apoptosis (16, 17). To con-
firm previous findings in our experimental setting, we measured specific cell death upon stimulation of SKW6.4 cells with different amounts of LZ-CD95L (Fig. 1A). SKW6.4 cells were efficiently undergoing apoptosis upon addition of LZ-CD95L as described previously. Using Western blot analysis, we observed processing of pro-caspase-8a/b (p55/p53) to its cleavage products: p43/p41 and p18 (Fig. 1B). This was accompanied by cleavage of pro-caspase-3, which is a reported substrate of caspase-8 in SKW6.4 cells (12, 18). This cleavage event indicates not only processing of pro-caspase-8 but also its catalytic activation. Caspase-3 activation, as judged by the processing of pro-caspase-3 and cleavage of the caspase-3 substrate, PARP, occurred shortly after caspase-8 activation (Fig. 1B) (19). Interestingly, we could detect that the strength of caspase activation depends on the amount of LZ-CD95L and takes places from minutes to hours after stimulation. Activation of caspases-8 and -3 peaked within 30 min upon stimulation with a high concentration of LZ-CD95L (1 μg/ml), within hours upon stimulation with the lower amounts of LZ-CD95L (100 ng/ml), and weak processing of pro-caspase-8/3 to their cleavage products was detectable only at the 3-h time point upon stimulation with even lower amounts of LZ-CD95L (10 ng/ml).

We also observed that the strength of caspase activation depends on the amount of LZ-CD95L using caspase-8 and -3 fluorescent substrates Z-IETD-AFC and Z-DEVD-AFC, respectively (Fig. 1, C–F). In contrast to the caspase activation at the CD95 DISC reported to occur within seconds after stimulation, caspase activation in the cytosol took place hours after stimulation. It should be noted that because of the lack of absolute specificity of Z-IETD-AFC, caspase-3 activity might also contribute to the increase in caspase-8 activity measured using Z-IETD-AFC. However, the data from Western blot analysis of caspase-8 and -3 activation (Fig. 1B) show that caspase-3 activation peaks shortly after caspase-8 activation supporting the data of caspase activity assays.

Interestingly, it has been reported that the CD95 DISC is formed within seconds after CD95 stimulation, followed by caspase-8 activation at the DISC complex (11, 17, 20). Furthermore, it has been shown that CD95 DISC formation at the membrane peaks minutes after stimulation and that the amount of the active CD95 DISC is markedly reduced 1 h after stimulation (21). The kinetics of DISC formation contradict our results (Fig. 1) as follows: caspase-8 processing/activation in the lysates occurs within hours after stimulation. This suggests that caspase-8 activation may also take place downstream of the DISC, which raises the question whether there are other cytosolic complexes formed upon CD95 stimulation that may activate caspase-8.

A Novel DED Protein-containing Complex Is Formed upon CD95 Stimulation—To address the issue whether there are other complexes apart from the DISC that contain pro-caspase-8, SKW6.4 cells were stimulated with LZ-CD95L followed by caspase-8 immunoprecipitation (Fig. 2A, right panel). Caspase-8 immunoprecipitation (C8-IP) was performed using the C15 antibodies, directed against the C terminus of pro-
caspase-8 (14), which was followed by Western blot analysis. Without CD95 stimulation, we observed c-FLIPL associated with procaspase-8a/b. Upon CD95 stimulation we could co-immunoprecipitate FADD, procaspase-8a/b, c-FLIP<sub>L</sub>, as well as cleavage products of procaspase-8 and c-FLIP (Fig. 2, right panel). Interestingly, we did not detect CD95 in this co-immunoprecipitation. All DED proteins involved in CD95 signal transduction in lymphocytes were detected in this complex as follows: FADD, procaspase-8a/b, both long and short isoforms of c-FLIP, c-FLIPL<sub>R</sub>, and c-FLIP<sub>K</sub>, respectively. c-FLIP<sub>R</sub> was shown to be one of two short c-FLIP isoforms present in SKW6.4 cells in our previous work (22). As a positive control we used DISC immunoprecipitation (CD95-IP), which we performed using agonistic anti-APO-1 antibodies in accordance with the method applied in our previous studies (16, 17, 23) (Fig. 2A, left panel). As described before, we could detect CD95, procaspase-8, FADD, and c-FLIP at the DISC (Fig. 2A, left panel). Thus, the analysis of C8-IP indicated that there is a novel DED protein-containing complex formed upon CD95 stimulation that does not contain CD95. Procaspase-8, c-FLIP, and FADD were found to be present in this complex, which we termed complex II.

To further confirm these findings, SKW6.4 cells were stimulated with LZ-CD95L. Then we performed immunoprecipitation using anti-FADD antibodies 1C4 (FADD-IP), directed against the C terminus of FADD (13). C8-IP, CD95-IP, and protein A-IP were run in parallel (Fig. 2B). Using anti-FADD antibodies, we also observed CD95 stimulation-dependent association of the DED-containing proteins, procaspase-8, FADD, and c-FLIP.

To examine the kinetics of complex II formation, we stimulated SKW6.4 cells with LZ-CD95L and analyzed DISC and complex II composition by Western blot (Fig. 2C). Complex II was detected shortly after DISC formation. Four hours after stimulation, all DED proteins were found at the complex II as follows: procaspase-8, FADD, and c-FLIP (Fig. 2C). Interestingly, the processing of c-FLIP<sub>L</sub> to p43-FLIP<sub>L</sub> at complex II was delayed, whereas at the DISC c-FLIP<sub>L</sub> was almost completely processed to p43-FLIP<sub>L</sub> immediately after stimulation. Furthermore, the kinetics of active caspase-8 (p18) generation peaked simultaneously in complex II and in total cellular lysates. This suggests that complex II contributes to the activation of caspase-8 in

caspase-8 (14), which was followed by Western blot analysis. Without CD95 stimulation, we observed c-FLIPL<sub>R</sub> associated with procaspase-8a/b. Upon CD95 stimulation we could co-immunoprecipitate FADD, procaspase-8a/b, c-FLIP<sub>L</sub>, as well as cleavage products of procaspase-8 and c-FLIP (Fig. 2A, right panel). Interestingly, we did not detect CD95 in this co-immunoprecipitation. All DED proteins involved in CD95 signal transduction in lymphocytes were detected in this complex as follows: FADD, procaspase-8a/b, both long and short isoforms of c-FLIP, c-FLIPL<sub>R</sub>, and c-FLIP<sub>K</sub>, respectively. c-FLIP<sub>R</sub> was shown to be one of two short c-FLIP isoforms present in SKW6.4 cells in our previous work (22). As a positive control we used DISC immunoprecipitation (CD95-IP), which we performed using agonistic anti-APO-1 antibodies in accordance with the method applied in our previous studies (16, 17, 23) (Fig. 2A, left panel). As described before, we could detect CD95, procaspase-8, FADD, and c-FLIP at the DISC (Fig. 2A, left panel). Thus, the analysis of C8-IP indicated that there is a novel DED protein-containing complex formed upon CD95 stimulation that does not contain CD95. Procaspase-8, c-FLIP, and FADD were found to be present in this complex, which we termed complex II.

To further confirm these findings, SKW6.4 cells were stimulated with LZ-CD95L. Then we performed immunoprecipitation using anti-FADD antibodies 1C4 (FADD-IP), directed against the C terminus of FADD (13). C8-IP, CD95-IP, and protein A-IP were run in parallel (Fig. 2B). Using anti-FADD antibodies, we also observed CD95 stimulation-dependent association of the DED-containing proteins, procaspase-8, FADD, and c-FLIP.

To examine the kinetics of complex II formation, we stimulated SKW6.4 cells with LZ-CD95L and analyzed DISC and complex II composition by Western blot (Fig. 2C). Complex II was detected shortly after DISC formation. Four hours after stimulation, all DED proteins were found at the complex II as follows: procaspase-8, FADD, and c-FLIP (Fig. 2C). Interestingly, the processing of c-FLIP<sub>L</sub> to p43-FLIP<sub>L</sub> at complex II was delayed, whereas at the DISC c-FLIP<sub>L</sub> was almost completely processed to p43-FLIP<sub>L</sub> immediately after stimulation. Furthermore, the kinetics of active caspase-8 (p18) generation peaked simultaneously in complex II and in total cellular lysates. This suggests that complex II contributes to the activation of caspase-8 in total cellular lysates.

Additionally, we examined the early kinetics of complex II and DISC formation within minutes after stimulation (Fig. 2D). After 2 min of stimulation, we can already co-immunoprecipitate procaspase-8, FADD, and c-FLIP in complex II lacking CD95. Thus, it seems that complex II formation occurs too fast to detect the initial steps using standard immunoprecipitation procedures.

**Complex II Is Distinct from the CD95 DISC**—Next, we aimed to rule out the possibility that complex II is a part of the CD95 DISC. Therefore, we depleted the lysates from the CD95 DISC and subsequently performed C8-IP (Fig. 3). SKW6.4 cells were stimulated with LZ-CD95L, followed by CD95-IP (Fig. 3, lane 1) and C8-IP (Fig. 3, lane 6), which were done in parallel. To ensure the quantitative depletion of the DISC from the lysates, supernatants from CD95-IP (Fig. 3, lane 1) were subjected to the second CD95-IP (Fig. 3, lane 2). This step was repeated for the third time (Fig. 3, lane 3). The amount of CD95 DISC left in the lysates was significantly reduced already after the first CD95-IP (Fig. 3, lane 2). Following the third CD95-IP, the
lysates were precleared twice with protein A beads to remove anti-APO-1 antibodies (Fig. 3, lanes 4 and 5). Thus, in the lysates depleted from the DISC, we observed the association of FADD, c-FLIP, and procaspase-8 in the absence of CD95 (Fig. 3, lanes 7). This experiment shows that the DISC is indeed different from complex II, the latter being composed of FADD, c-FLIP, and procaspase-8.

**Complex II Is Formed in Both Type I and Type II Cell Lines**

Next, we investigated the formation of complex II in other T and B cell lines. We stimulated T cell lines J16 and HUT78 as well as B lymphoblastoid cells SKW6.4, BJAB, and Raji with LZ-CD95L. Stimulation was followed by CD95-IP using agonistic anti-APO-1 antibodies and C8-IP using C15 antibodies (Fig. 4A). In all investigated T and B cell lines, we could co-immunoprecipitate DED-containing proteins procaspase-8a/b, FADD, and c-FLIPL/S/R upon stimulation with LZ-CD95L. Procaspase-8a/b and c-FLIPL in complex II were processed to their cleavage products in all cell lines. Procaspase-8a/b was processed to p43/p41 and to the active subunit p18. C-FLIPL was processed to p43-FLIPL. Notably, SKW6.4, HUT78, Raji, and BJAB cells belong to type I cells, and J16 cells belong to type II cells (12). Thus, we observe complex II formation in both type I and type II cells.

To further verify these data, the panel of cell lines (SKW6.4, HUT78, Raji, BJAB, and JA3) was stimulated with LZ-CD95L for 30 min and then subjected to FADD-IP in parallel to C8-IP (Fig. 4B). Using FADD-IP, we also observed association of all DED proteins upon CD95 stimulation in both type I and type II cells. Procaspase-8 and c-FLIPL in complex II were also processed to their cleavage products. Procaspase-8 was processed to p43/p41 and to the active subunit p18, indicating that active caspase-8 is generated in the complex II. c-FLIPL was processed to p43-FLIPL. Thus, we have shown that complex II formation is a general phenomenon observed in several T and B cell lines upon CD95 stimulation.

**Complex II Can Amplify Caspase Activation**

To unravel the function of complex II, we investigated whether complex II can cleave procaspase-3 and thereby amplify caspase activation. We have observed active caspase-8 (p18) in complex II, which might possess catalytic function (Figs. 2–4). Therefore, we immunoprecipitated complex II from the lysates that were immunodepleted from the DISC as we described earlier in Fig. 3. In summary, SKW6.4 cells were stimulated with LZ-CD95L, which was followed by several steps of CD95-IPs, which, in turn, was followed by C8-IP. Afterward, *in vitro* translated procaspase-3 was added to the C8-IP. The processing of procaspase-3 was analyzed by Western blot (Fig. 5A). We have observed the generation of the cleavage product of procaspase-3, p19 upon addition of complex II. The processing of procaspase-3 was rather weak, however. Therefore, we used an *in vitro* system, and we probably did not have optimal concentrations of complex II and procaspase-3 leading to more efficient processing of procaspase-3. Therefore,
we concluded that complex II might process procaspase-3, which would lead to the amplification of caspase activation.

To further analyze whether complex II forms a bigger complex involving other pro-apoptotic components such as procaspase-3 and procaspase-9, we checked procaspase-3/9 presence in complex II using Western blot (Fig. 5, B and C). However, we did not detect either procaspase-3 or procaspase-9 in complex II. Hence, caspase-8 is the main caspase contributing to pro-apoptotic catalytic activity of complex II.

As CD95 stimulation was also reported to induce NF-κB in parallel to apoptosis induction, we analyzed the activation of this pathway upon LZ-CD95L stimulation. We observed the activation of NF-κB in SKW6.4 cells as judged by phosphorylation of IκBα (Fig. 5D). To analyze the possible involvement of complex II in the transduction of pro-survival signals, we checked complex II immunoprecipitated from SKW6.4 for the presence of TRAF2 and RIP1, which were reported to be involved in the CD95-mediated NF-κB activation (24, 25). However, we could not detect TRAF2 and RIP1 in the DISC and complex II using Western blot analysis (Fig. 5E). As our analysis involves only one cell line, SKW6.4 cells, we could not exclude that TRAF2 and RIP1 might be components of these complexes. The role of complex II in CD95-mediated pro-survival signaling should be further analyzed in future studies and might also involve new unidentified proteins in this complex.

**DISCUSSION**

In this study we describe a new complex, which includes the following DED-containing proteins: FADD, c-FLIP, and procaspase-8, which are the main components of the CD95 DISC. This complex is formed upon stimulation of CD95; however, it does not contain the death receptor (Fig. 6). The main function of complex II in CD95-induced apoptosis is likely the activation of procaspase-8 and amplification of caspase activation.

The mechanism of complex II formation remains to be determined. It was reported that upon stimulation CD95 undergoes clustering at the cellular membrane. Clustering of CD95 involves several steps. CD95 DISC formation is accompanied by building of stable microaggregates (26–28). This is followed by formation of SPOTS (signaling protein oligomeric transduction structures) leading to receptor clustering into large platforms (29). Recently it was shown that FADD plays an important structural role as a linker for these platforms (30, 31). Because of modification or conformational changes at the membrane, the cytosolic part of this platform comprising DED proteins might translocate to the cytoplasm and build a second cytosolic complex, complex II.

Our study suggests that in complex II procaspase-8 molecules are brought into close proximity, which is followed by their activation (32, 33). We have observed the formation of active caspase-8 (p18) in complex II using both FADD-IP and C8-IP (Figs. 2–4). This provides an indication that procaspase-8 undergoes autocatalytic processing generating active caspase-8 in complex II. Furthermore, we observed that complex II can process procaspase-3 in vitro. These data support the hypothesis that complex II indeed amplifies the caspase activation in the cytosol.

Another mechanism of amplification of the CD95-mediated death signal in the cytosol has recently been proposed. This mechanism involves the internalization of the CD95 DISC in type I cells (26). We clearly do not see CD95 in complex II (Figs. 2 and 3) and also observe complex II formation in type II cells, e.g., JA3 and J16 cells (Fig. 4). Thus, we show that the mechanism of complex II formation is independent of the mechanism of CD95 internalization. The possibility of cross-talk between these two pathways has to be addressed in future studies.

Interestingly, there is growing evidence that a number of cytosolic complexes formed upon death receptor stimulation are essential for life/death decisions. The formation of complex II comprising procaspase-8, c-FLIP, FADD, RIP, TRADD, and TRAF2 was described for tumor necrosis factor signaling (34). The main function of this complex involves the transduction of the death signal, e.g., caspase-8 activation. Recently, it has been demonstrated that upon TRAIL stimulation the same molecules are involved in the formation of a secondary complex, and this complex is essential for the transduction of both life and...
death signals (35). Interestingly, Jin and El Deiry (36) have suggested the possibility of complex II formation in CD95 signaling. Here we demonstrate the formation of a secondary DED-protein-containing complex, which occurs upon CD95 stimulation. This complex likely plays a central role in the cytosolic activation of procaspase-8 or might also contain components leading into pathways of proliferation. The understanding of the role of complex II in life and death pathways is a matter of future studies.

There have been a number of reports demonstrating that upon T cell receptor stimulation procaspase-8 and FADD form a cytosolic complex together with Malt1 and Bcl-10, playing an important role in the activation of NF-κB (37). Furthermore, it has been reported that catalytic activity of procaspase-8 is required for NF-κB activation. Recently, it was also demonstrated that in monocytes undergoing macrophage differentiation, procaspase-8 forms an atypical complex together with FADD and RIP. This complex contributes to the regulation of NF-κB activity and, subsequently, to the process of macrophage differentiation (38). Thus, there can be two platforms for caspase-8 activation in the cytosolic complexes as follows: one in the death induction in complex II and another one in the proliferation-inducing Bcl-10-Malt1 complex. Elucidation of the stoichiometry and composition of these complexes may certainly shed light on the mechanism of caspase-8 activation in both pathways and provide more understanding of the life and death decisions made in the cell.

Taken together, we have described a novel caspase-8 activating complex formed upon CD95 stimulation. We suggest that life/death decision making takes place not only at membrane complexes but that cytosolic complexes are also essential for this decision.

Acknowledgments—We thank Dr. Dirk Brenner for critical comments, Petra Richter for help with experiments, and Heidi Saunter for excellent secretary work.

REFERENCES

1. Krammer, P. H. (2000) Nature 407, 789–795
2. Ashkenazi, A., and Dixit, V. M. (1998) Science 281, 1305–1308
3. Lavrik, I., Golk, A., and Krammer, P. H. (2005) J. Cell Sci. 118, 265–267
4. Suda, T., Takahashi, T., Golstein, P., and Nagata, S. (1993) Cell 75, 1169–1178
5. Trauth, B. C., Klas, C., Peters, A. M., Matzku, S., Moller, P., Falk, W., Debatin, K. M., and Krammer, P. H. (1989) Science 245, 301–305
6. Peter, M. E., and Krammer, P. H. (2003) Cell Death Differ. 10, 26–35
7. Lavrik, I. N., Golk, A., and Krammer, P. H. (2005) J. Clin. Investig. 115, 2665–2672
8. Sprick, M., Rieser, E., Stahl, H., Grosse-Wilde, A., Weigand, M., and Walczak, H. (2002) EMBO J. 21, 4520–4530
9. Muzio, M., Chinnaiyan, A. M., Kischkel, F. C., O’Rourke, K., Shevchenko, A., Ni, J., Scaffidi, C., Berset, J. D., Zhang, M., Gentz, R., Mann, M., Krammer, P. H., Peter, M. E., and Dixit, V. M. (1996) Cell 85, 817–827
10. Scaffidi, C., Schmitz, I., Krammer, P. H., and Peter, M. E. (1999) J. Biol. Chem. 274, 1541–1548
11. Medema, J. P., Scaffidi, C., Kischkel, F. C., Shevchenko, A., Mann, M., Krammer, P. H., and Peter, M. E. (1997) EMBO J. 16, 2794–2804
12. Scaffidi, C., Fulda, S., Srinivasan, A., Friesen, C., Li, F., Tomasselli, K. I., Debatin, K. M., Krammer, P. H., and Peter, M. E. (1998) EMBO J. 17, 1675–1687
13. Scaffidi, C., Volkland, J., Blomberg, I., Hoffmann, I., Krammer, P. H., and Peter, M. E. (2000) J. Immunol. 164, 1236–1242
14. Scaffidi, C., Medema, J. P., Krammer, P. H., and Peter, M. E. (1999) Cell Death Differ. 6, 821–822
15. Lavrik, I., Krueger, A., Schmitz, I., Baumann, S., Weyd, H., Krammer, P. H., and Kirchhoff, S. (2003) Cell Death Differ. 10, 144–145
16. Stenmicke, H. R., Jurgensmeier, J. M., Shin, H., Deveraux, Q. Q., Wolf, B. B., Yang, X., Zhou, Q., Ellery, H. M., Ellery, L. M., Bredesen, D., Green, D. R., Reed, J. C., Froelich, C. J., and Salvesen, G. S. (1998) J. Biol. Chem. 273, 27084–27090
17. Tewari, M., Quan, L. T., O’Rourke, K., Desnoyers, S., Zeng, Z., Beidler, D. R., Poirier, G. G., Salvesen, G. S., and Dixit, V. M. (1995) Cell 81, 801–809
18. Kischkel, F. C., Bellbardt, S., Behrmann, I., Germer, M., Paulita, M., Krammer, P. H., and Peter, M. E. (1995) EMBO J. 14, 5579–5588
19. Golks, A., Brenner, D., Schmitz, I., Watzl, C., Krueger, A., Krammer, P. H., and Lavrik, I. N. (2006) Cell Death Differ. 13, 489–498
20. Golks, A., Brenner, D., Fritsch, C., Krammer, P. H., and Lavrik, I. N. (2005) J. Biol. Chem. 280, 14507–14513
21. Krueger, A., Schmitz, I., Baumann, S., Krammer, P. H., and Kirchhoff, S. (2001) J. Biol. Chem. 276, 20633–20640
22. Kreuz, S., Siggand, D., Rumpf, J., Samel, D., Leverkus, M., Janssen, O., Hacker, G., Dittrich-Breiholz, O., Kracht, M., Scheurich, P., and Wajant, H. (2004) J. Cell Biol. 166, 369–380
23. Siggand, D., Klose, S., Zhou, D. H., Baumann, B., Roder, C., Kalthoff, H., Wajant, H., and Trauzold, A. (2007) Cell. Signal. 19, 1172–1184
24. Lee, K. H., Feig, C., Tchikov, V., Schickel, R., Hallas, C., Schutz, S., Peter, M. E., and Chan, A. C. (2006) EMBO J. 25, 1009–1023
25. Algeciras-Schimnich, A., Shen, L., Barnhart, C. B., Murmann, A. E., Burkhardt, J. K., and Peter, M. E. (2002) Mol. Cell. Biol. 22, 207–220
26. Feig, C., Tchikov, V., Schutz, S., and Peter, M. E. (2007) EMBO J. 26, 221–231
27. Siegel, R. M., Muppidi, J. R., Sarker, M., Lobito, A., Jen, M., Martin, D., Straus, E. S., and Lenardo, M. J. (2004) J. Biol. Cell 167, 735–744
28. Carrington, P. E., Sandu, C., Wei, Y., Hill, J. M., Morisawa, G., Huang, T., Gavathiotis, E., Wei, Y., and Werner, M. H. (2006) Mol. Cell 22, 599–610
29. Sandu, C., Morisawa, G., Wegorzewska, I., Huang, T., Arecchiga, A. F., Hill, J. M., Kim, T., Walsh, C. M., and Werner, M. H. (2006) Cell Death Differ. 13, 2052–2061
30. Salvesen, G. S., and Dixit, V. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 10964–10967
31. Muzio, M., Stockwell, B. R., Stennicke, H. R., Salvesen, G. S., and Dixit, V. M. (1998) J. Biol. Chem. 273, 2926–2930
32. Michele, O., and Tsopp, J. (2003) Cell 114, 181–190
33. Varfolomeev, E., Macek, H., Sharp, D., Lawrence, D., Renz, M., Vucic, D., and Ashkenazi, A. (2005) J. Biol. Chem. 280, 40599–40608
34. Ivan, Z., and El Deiry, W. S. (2006) Mol. Cell. Biol. 26, 8136–8148
35. Su, H., Bidere, N., Zheng, L., Cubre, A., Sakai, K., Dale, J., Salmena, L., Hakem, R., Straus, S., and Lenardo, M. (2005) Science 307, 1465–1468
36. Rebe, C., Cathelin, S., Launay, S., Filomeno, R., Prevotat, L., L’Ollivier, C., Gyan, E., Micheau, O., Grant, S., Dubart-Kupperschmitt, A., Fontenay, M., and Solary, E. (2007) Blood 109, 1442–1450