CD95 ligand (CD95L) triggers a rapid formation of reactive oxygen species (ROS) as an upstream event of CD95 activation and apoptosis induction in rat hepatocytes. This ROS response was sensitive to inhibition by diphenyleneiodonium, apocynin, and neopterin, suggestive of an involvement of NADPH oxidases. In line with this, hepatocytes expressed mRNAs not only of the phagocyte gp91phox (Nox 2), but also of the homologs Nox 1 and 4 and Duox 1 and 2, as well as the regulatory subunit p47phox. gp91phox (Nox 2) and p47phox were also identified at the protein level in rat hepatocytes. CD95L induced within 1 min ceramide formation and serine phosphorylation of p47phox, which was sensitive to inhibitors of sphingomyelinase and protein kinase Cζ (PKCζ). These inhibitors and p47phox protein knockdown inhibited the early CD95L-induced ROS response, suggesting that ceramide and PKCζ are upstream events of the CD95L-induced Nox/Duox activation. CD95L also induced rapid activation of the Src family kinase Yes, being followed by activation of c-Src, Fyn, and c-Jun-N-terminal kinases (JNK). Only Yes and JNK activation were sensitive to N-acetylcysteine, inhibitors of NADPH oxidase, PKCζ, or sphingomyelinase, indicating that the CD95L-induced ROS response is upstream of Yes and JNK but not of Fyn and c-Src activation. Activated Yes rapidly associated with the epidermal growth factor receptor (EGFR), which became phosphorylated at Tyr1173 but not at Tyr1045. Activated EGFR then triggered an AG1478-sensitive CD95-tyrosine phosphorylation, which was a signal for membrane targeting of the EGFR/CD95 complex, subsequent recruitment of Fas-associated death domain and caspase 8, and apoptosis induction. All of these events were significantly blunted by inhibitors of sphingomyelinase, PKCζ, NADPH oxidases, Yes, or EGFR-tyrosine kinase activity and after protein knockdown of either p47phox, Yes, or EGFR.

The data suggest that CD95L-induced apoptosis involves a sphingomyelinase- and PKCζ-dependent activation of NADPH oxidase isoforms, which is required for Yes/EGFR/CD95 interactions as upstream events of CD95 activation.

CD95<sup>1</sup> (APO-1/Fas) belongs to the death receptor family and plays an important role in apoptosis induction in many cell types. In liver, CD95 can be activated after ligation with its natural ligand (CD95L); however, CD95 can also be activated in a ligand-independent way, for example by hydrophobic bile acids or hyperosmotic cell shrinkage (1, 2). Ligand-dependent and -independent CD95 activation in hepatocytes is a complex process, which finally results in the formation of the death-inducing signaling complex (DISC) and activation of the initiator caspase 8, which then triggers a variety of complexly regulated downstream events, which may finally result in apoptotic cell death. In hepatocytes, proapoptotic stimuli such as CD95L, hydrophobic bile acids, or hyperosmotic cell shrinkage induce a rapid oxidative stress response, which triggers ligand-independent activation of the epidermal growth factor receptor (EGFR) and subsequent c-Jun-N-terminal kinase (JNK)-dependent association of the EGFR with CD95 within 30 min (1, 2). Following EGFR-catalyzed tyrosine phosphorylation of the CD95, the EGFR/CD95 protein complex is targeted in a microtubule-dependent way to the plasma membrane, where formation of the DISC occurs (3). The mechanisms underlying CD95L-triggered EGFR activation are not fully understood; however, EGFR activation in response to other proapoptotic stimuli involves an antioxidant-sensitive activation of Yes as upstream event (4, 5).

Yes, like Fyn and c-Src, are members of the Src kinase family, which are ubiquitously expressed (for a review, see Refs. 6 and 7). In rat hepatocytes, c-Src participates in integrin-dependent osmosignaling (8), whereas Yes is important for apoptosis induction through the CD95 system by CD95 ligand-independent stimuli (4, 5). In nonhepatic cell types, hyperosmotic Fyn activation results in an increased phosphorylation of caveolin (9) and cortactin (10). The mechanisms of how CD95L and other proapoptotic stimuli trigger the oxidative stress response in hepatocytes remained unclear. Like tumor necrosis factor-α, proapoptotic bile acids were suggested to induce oxygen radical formation by mitochondria (11–14); however, this may represent a downstream conse-

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<sup>1</sup>The abbreviations used are: CD95, CD95 receptor; Fas, APO-1; CD95L, CD95 ligand; CD95-Tyr-P, CD95-tyrosine phosphorylation; CFP, cyan fluorescent protein; CM-H<sub>2</sub>DCFDA, 5-(and-6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate; DISC, death-inducing signaling complex; DPI, diphenyleneiodonium chloride; EGFR, epidermal growth factor receptor; FADD, Fas-associated death domain; Huh7, human hepatoma cell line 7; JNK, c-Jun N-terminal kinase; PKC, protein kinase C; PKCζ inhibitor, cell-permeable myristoylated PKCζ pseudosubstrate; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated X-dUTP nick end labeling; YFP, yellow fluorescent protein; RT, reverse transcription; PBS, phosphate-buffered saline; FRET, fluorescence resonance energy transfer; DCF, 2′,7′-dichlorofluorescein; HSC, hepatic stellate cell(s); NAC, N-acetylcysteine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
quence but not the cause of CD95 activation. In phagocytes, reactive oxygen species are generated by an NADPH oxidase complex with its catalytic moiety gp91phox, which is activated by assembly with regulatory proteins such as p47phox, p67phox, and Rac (15–18). Recently, membrane oxidases similar to the phagocytic NADPH oxidase complex were found also in nonphagocytic cell types. These homologs of gp91phox are called Nox and Duox, with the classical phagocytic gp91phox being termed Nox 2. The isoform Nox 3 was found in fetal kidney, Nox 1 is predominantly expressed in the colon, and Nox 5 is predominantly expressed in spleen and fetal tissues (19, 20). Duox 1 and 2 reflect high molecular mass gp91phox homologs with an N-terminal peroxidase domain in addition to the C-terminal NADPH oxidase activity. Duox 1 was found primarily in the thyroid gland, whereas Duox 2 is also found in the intestine and the colon. Nox and Duox proteins were suggested to participate in signal transduction addition to the C-terminal NADPH oxidase activity. Duox 1 and 2 reflect high molecular mass and Nox 5 is predominantly expressed in spleen and fetal tissues (19, 20). Duox 1 and 2 indicate oxidative stress by rapid oxidative stress signal in response to proapoptotic stim-

**EXPERIMENTAL PROCEDURES**

*Materials—The materials used were purchased as follows: collagens and terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay from Roche Molecular Sciences, Inc. (Indianapolis, IN); medium, C(2)S-dihydroceramide, desipramine, and A99444, 14(15 +)-neotinin from Sigma; penicillin/streptomycin from Biochrom (Berlin, Germany); fetal calf serum, Dulbecco’s modified Eagle’s medium/nut mix F-12, pTOPO-TA vector, and Lipofectamine 2000 from Invitrogen; G06850 from Tocris Cookson Ltd. (Bristol, UK); l-JNK1 (c-Jun N-terminal kinase inhibitor) from Alexis Biochemicals (San Diego, CA); diphenyletheno-

**Table 1**

| Inhibitor               | Target          | Reference |
|-------------------------|-----------------|-----------|
| A9Y944                  | Sphingomyelinase | 41        |
| Desipramine             | Sphingomyelinase | 42        |
| Diphenyletheno-
| nium                     | NADPH oxidase   | 77        |
| Apocynin                | NADPH oxidase   | 60        |
| (H9252)-H9254          | NADPH oxidase   | 79        |
| N-Acetylcycteine        | Scavenges reactive oxygen intermediates | 74 |
| myr-PKCζ-pseudosubstrate | PKCζ           | 79        |
| Chelerythrine           | PKCa, -β, -γ, -δ, -ε, -ζ | 80 |
| G06850                  | PKCa, -β, -δ, -ε | 45        |
| G06876                  | PKCa, -β, -μ | 45        |
| Ro-32-0432              | PKCa, -β, -δ, -γ, -ε | 46 |
| SU6506                  | Src family kinases (Yes, Fyn, c-Src) | 49 |
| PP-2                    | Src family kinases (c-Src, Fyn, Lck, Hck) | 50 |
| Herbinicin A            | Src family kinase c-Src | 51 |
| l-JNK1 (c-Jun N-terminal kinase inhibitor) | 81 |
| Genistein               | Unspecific tyrosine kinase | 82 |
| AG1478                  | EGFR-tyrosine kinase activity | 83 |

bit anti-gliarial fibrillary acidic protein from Sigma; mouse anti-ED-2 from Serotec (Düsseldorf, Germany); and horseradish peroxidase-conjugated anti-mouse IgG and anti-rabbit IgG from Bio-Rad. All other chemicals were from Merck at the highest quality available.

**Cell Preparation and Culture—Hepatocytes, Kupffer cells, and hepatic stellate cells** were isolated from livers of male Wistar rats, fed ad libitum with a standard diet, by a collagenase perfusion technique as described previously (1, 2, 24). Aliquots of 1.5 × 10⁸ hepatocytes were plated on collagen-coated 6-well culture plates (Falcon, Heidelberg, Germany) and cultured as published recently (1, 2, 24) for 24 h, unless indicated otherwise, before the experiments were started. The viability of the hepatocytes was more than 95% as assessed by trypan blue exclusion. Immunochemistry staining of the 24-h hepatocyte culture was prepared as recently published (25). Human monocytes were obtained from peripheral blood samples using BD Vacutainer CPT Cell Preparation Tubes (BD Biosciences) according to the manufacturer’s recommendations.

**Human hepatoma cell line 7 (Huh7)** cells (26) were cultured in a humidified 5% CO₂ atmosphere at 37 °C in Dulbecco’s modified Eagle’s medium/nut mix F-12 supplemented with 10% fetal calf serum and 1% gentamycin. Rat peritoneal macrophages were prepared as recently published (25). Human monocytes were grown to 70% confluence before transient transfection using expression vectors of the CD95-YFP and EGFR-CFP fusion proteins supplemented with Lipofectamine 2000 with the manufacturer’s recommendations.

**Plasmid Construction—** The nucleotide sequence encoding CD95 was amplified by reverse transcription (RT)-PCR using HepG2 mRNA and ligation into the pTOPO-TA vector (Invitrogen). After the introduction of restriction sites and removal of the stop codon by PCR, the fragment was inserted into pEYF-PN1 (Clontech) to create a fusion protein with YFP linked to the C terminus. The EGFR-CFP-construct was obtained by replacing GFP of F7 erb B1-EGFP (kindly provided by Prof. Arndt-Jovin, Ph.D. (27)) with CFP of pECFP-N1 (Clontech). After the introduction of restriction sites and removal of the stop codon by PCR, the fragment was inserted into pEYF-PN1 (Clontech) to create a fusion protein with YFP linked to the C terminus. The EGFR-CFP-construct was obtained by replacing GFP of F7 erb B1-EGFP (kindly provided by Prof. Arndt-Jovin, Ph.D. (27)) with CFP of pEFP-PN1 (Clontech). All constructs were confirmed by sequencing (MWG Biotechnology, Ebersberg, Germany).

**RT-PCR—** Total RNA was isolated from cultured rat hepatocytes (1 and 4 days of culture), HSC (1 and 14 days of culture), Kupffer cells, and fetal rat kidney using the peqGold TriFast kit (peqLab Biotechnologie GmbH, Erlangen, Germany). Purified RNA was quantified by spectrophotometry. The first strand cDNA was made from 1 μg of total RNA/25 μl reaction volume with the RevertAid H Minus First Strand cDNA synthesis kit (Fermentas GmbH, St. Leon-Rot, Germany). The PCR was...
performed using the 2× PCR Master Mix (Fermentas GmbH, St. Leon-Rot, Germany) as well as the following primers: for rat p47phox, sense (5'-GCC CAA AGA TGG CAA GAA TA-3') and antisense (5'-TGG CAT CCA AAT AGT GC-3'); for rat NOX1, sense (5'-GAC GAA TGG CTT GAT CTT GTG-3') and antisense (5'-CTC CCA AAG GAG GGT TTC TGC TGT-3'); for rat NOX2, sense (5'-TCA AGT GTC CCC AGG TAG CC-3') and antisense (5'-CTC CAT TGG TAC CAA AGG-3'); for rat NOX3, sense (5'-AAT CAG AGA GTC TGC GTG CAC T-3') and antisense (5'-ATA CGG AGT GGT GGG TGG CTA-3'); for rat NOX4, sense (5'-AGG AGT CCA TTG GAG TCA TGC G-3') and antisense (5'-TGG AGG TGT CAC GAG TAT CCA GGA-3') and antisense (5'-AACA CAA TGG CTT GGA TGA AT-3'); for rat β-actin as control, sense (5'-GCC GTA TAG TTC GGC CAC-3') and antisense (5'-CCA TAG TCA TGA AAG AC-3') and antisense (5'-AA-CAA TGG CCT GGA TGA AT-3'); for human NOX1, sense (5'-TATA ACA CCA GCC TGA TCC TG-3') and antisense (5'-CTG GAG AGA GAT GAG GCA AG-3'); for human NOX2, sense (5'-GAA CGT CTT CTT TCT GTG GC-3') and antisense (5'-GTT AGT AGA CTA CCA GTG ATG C-3'); for human NOX3, sense (5'-AGG CCC TGT GGT CTT GGA TGA-3') and antisense (5'-ACA TCT GGT CTT CTC ATG C-3'); for human NOX4, sense (5'-GAA GGG TGG CTG CTT TGG AT-3') and antisense (5'-AGG AGA AGT TGC GAT CTC G-3'); for human NOX5, sense (5'-GAG GAA GAG GAT GGA TGA TGC G-3') and antisense (5'-GGA AAA TCT GGC ACC ACA GC-3') and antisense (5'-TCT TCC ATT GTC TGA CCA GT-3'); and for human NOX1, sense (5'-TATA ACA CCA GCC TGA TCC TG-3') and antisense (5'-CTG GAG AGA GAT GAG GCA AG-3'); for human NOX2, sense (5'-GAA CGT CTT CTT TCT GTG GC-3') and antisense (5'-GTT AGT AGA CTA CCA GTG ATG C-3'); for human NOX3, sense (5'-AGG CCC TGT GGT CTT GGA TGA-3') and antisense (5'-ACA TCT GGT CTT CTC ATG C-3'); for human NOX4, sense (5'-GAA GGG TGG CTG CTT TGG AT-3') and antisense (5'-AGG AGA AGT TGC GAT CTC G-3'); for human NOX5, sense (5'-GAG GAA GAG GAT GGA TGA TGC G-3') and antisense (5'-GGA AAA TCT GGC ACC ACA GC-3') and antisense (5'-TCT TCC ATT GTC TGA CCA GT-3'). All primer sets were optimized on a real-time fluorescence PCR machine (MKG Biotech, Ebersberg, Germany) using a real-time PCR kit (ABgene, Esher, UK) and were used at a final concentration of 200 nmol/liter, and 5 μl of template cDNA was added per 25 μl reaction volume. PCR was conducted with a thermal cycler using standard protocols. Products of PCR were analyzed by 2% agarose gels and ethidium bromide staining. Fragments were excised, treated with the complementary DNA ladder MassRuler (low range, Fermentas GmbH, St. Leon-Rot, Germany). Obtained PCR products were confirmed by partial sequencing after separation from agarose gels (Wizard SV Gel and PCR Clean-Up System, Promega, Madison, WI).

p47phox, Yes, and EGFR Protein Knockdown—Antisense oligonucleotides directed to p47phox, the Src kinase family member Yes, or EGFR and the corresponding controls (i.e. nonsense oligonucleotides) were designed and manufactured by Biognostik (Göttingen, Germany). Immediately after hepatocytes were plated on collagen-coated culture plates (diameter, 6 cm; Falcon) at a density of 4 × 10⁶ cells/plate, either a 4 μmol/liter concentration of the respective oligonucleotides (non-sense, p47phox, or Yes antisense) or a mixture of 1× nonsense and EGFR antisense was added to the cultures. Oligonucleotides were modified with a cationic lipid (DOTMA) and were added on top of the cell monolayer. After 24 h, the treated cells were washed extensively and developed using enhanced chemiluminescent detection (Amersham Biosciences). Blots were exposed to X-Omat AR-5 film (Eastman Kodak Co.).

Immuno precipitation—Hepatocytes were cultured on collagen-coated culture plates (diameter, 10 cm; Falcon) at a density of 8 × 10⁶ cells/plate. They were harvested in lysis buffer as recently published (1, 2). Equal protein amounts (200 μg) of each sample were incubated for 2 h at 4 °C with a polyclonal rabbit anti-CD95, rabbit anti-p47phox, rabbit anti-Yes, or rabbit anti-Fyn antibody (dilution 1:100; Santa Cruz Biotechnology) in order to immunoprecipitate CD95, p47phox, Yes, or Fyn. Then 10 μl of protein A-agarose and 10 μl of protein G-agarose (Santa Cruz Biotechnology) were added and incubated at 4 °C overnight. Immunoprecipitates were washed 3 times as published recently (1, 2) and then transferred to Western blot analysis as described above. Activation of p47phox by serine phosphorylation was detected using an anti-phosphoserine antibody (28). The anti-phospho-Src family-Tyr418 antibody was used in order to detect activating phosphorylation of Yes or Fyn in the respective immunoprecipitates (29), whereas Yes or Fyn/EGFR association was detected using an anti-EGFR-antibody in the latter samples. EGFR, FADD, and caspase 8 association or tyrosine phosphorylation of the immunoprecipitated CD95 samples were detected by Western blot analysis using the respective antibodies (anti-EGFR, –FADD, –caspase 8, and –phosphotyrosine).

Subcellular Fractionation—Hepatocytes were cultured on collagen-coated culture plates (diameter, 10 cm; Falcon) at a density of 8 × 10⁶ cells/plate. Cells were lysed in a buffer containing 10 mmol/liter Tris, 30 mmol/liter mannitol, and 10 mmol/liter CaCl₂ (pH 7.5). After centrifugation of the samples (5 min, 1200 g), the supernatants were subjected to ultracentrifugation (35 min, 40,000 g) in order to separate the plasma membrane fraction (pellet) from the cytosolic compartment including intracellular endomembranes (membrane fraction). The latter samples were then again subjected to ultracentrifugation (2 h, 100,000 g) in order to separate the intracellular endomembranes (pellet) from the cytosolic compartment (supernatant). The latter fractions were then used as described above. For the membrane traffic of CD95 in primary rat hepatocytes, cells were cultured for 24 h on collagen-coated glass coverslips (diameter, 30 mm) in 6-well culture plates (Falcon). Permeabilized and nonpermeabilized cells were stained as published recently (1, 2), using a polyclonal rabbit anti-CD95 antibody (dilution 1:500 in PBS) and a secondary anti-rabbit Cy3-conjugated antibody. Cells were visualized using an Axiophot (Zeiss, Oberkochen, Germany), and pictures were taken with a 3CCD camera (Intas, Göttingen, Germany). Receptor membrane translocation was defined as the appearance of fluorescent spotting on the surface of the nonpermeabilized cells compared with the nonpermeabilized control cells (2). The latter samples were then again subjected to ultracentrifugation (2 h, 100,000 g) in order to separate cytosolic from membrane fractions, which were assayed for CD95 signal detectable in the cytosolic fraction, indicating a high efficiency of separation.

CD95 and PKCζ Membrane Translocation—For determination of membrane surface trafficking of CD95 in primary rat hepatocytes, cells were cultured for 24 h on collagen-coated glass coverslips (diameter, 30 mm) in 6-well culture plates (Falcon). Permeabilized and nonpermeabilized cells were stained as published recently (1, 2), using a polyclonal rabbit anti-CD95 antibody (dilution 1:500 in PBS) and a secondary anti-rabbit Cy3-conjugated antibody. Cells were visualized using an Axiophot (Zeiss, Oberkochen, Germany), and pictures were taken with a 3CCD camera (Intas, Göttingen, Germany). Receptor membrane translocation was defined as the appearance of fluorescent spotting on the surface of the nonpermeabilized cells compared with the nonpermeabilized control cells (2). The latter samples were then again subjected to ultracentrifugation (2 h, 100,000 g) in order to separate cytosolic from membrane fractions, which were assayed for CD95 signal detectable in the cytosolic fraction, indicating a high efficiency of separation.

CD95 translocation was also studied by detecting total CD95 amount in cytosolic and membrane fractions using Western blot analysis. For this, cells were plated on collagen-coated culture plates (Ø 10 cm) at a density of 8 × 10⁶ cells/plate and were lysed as centrifuged (35 min, 40,000 × g) in order to separate cytosolic from membrane fractions as recently published (1, 2). Total CD95 amount in the latter fractions was then detected by Western blotting as described above.

In order to detect PKCζ membrane translocation, cells were cultured for 24 h on collagen-coated glass coverslips (diameter, 30 mm) in 6-well culture plates. After treatment for 1 min with either control medium or CD95L (100 ng/ml), cells were fixed for 3 min using methanol (–20 °C) and then permeabilized using Triton X-100 (0.1% v/v) in PBS, 10 min, room temperature). Cells were washed briefly with PBS (4 °C) and then incubated with a rabbit anti-PKCζ antibody (1 h, 4 °C, 1:200 in PBS), washed off, and then stained with an anti-rabbit Cy3-conjugated antibody (1 h, 4 °C, 1:500 in PBS). Coverslips were mounted with diazabicyclo[2.2.2]octane 0.1% in glycerine/PBS (9:1), and cells were visualized using a Leica TCS-NT confocal laser-scanning microscope with a 10× air objective and a 40× oil objective on a Leica DM IRB inverted microscope (Bensheim, Germany).

Fluorescence Resonance Energy Transfer (FRET) Experiments—FRET was used in order to visualize CD95L-induced interactions between EGFR and CD95. For this purpose, HuH7 hepatoma cells were cotransfected with CD95-YFP and EGFR-CFP, as described in detail recently (3). Confocal pictures were taken using the LSM-510 META (Zeiss). All YFP/CFP cotransfections were detected using the META.
scanned, avoiding bleed-through of CPF in the YFP channel. CPF was excited with 405 nm, and YFP was excited with 514 nm (30). FRET efficiency was determined using LSM-Image-Examiner-3.1 software (Zeiss), and FITC pictures were normalized for the FRET efficiencies in the respective setting as indicated by the accompanying scale (FRET efficiency is given from blue/0 to red/255).

**Detection of Reactive Oxygen Species (ROS)—**Hepatocytes were seeded on collagen-coated 6-well culture plates (Falcon) and cultured 24 h for inhibitor experiments or 4 days for protein knockdown experiments, respectively. Cells were incubated with PBS containing 5 μmol/liter of CM-H₂DCFDA for 30 min at 37 °C and 5% CO₂. The oxidation of the nonfluorescent 2′,7′-dichlorodihydrofluorescein diacetate, also known as dichlorofluorescein diacetate, to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) is commonly used to detect the overall generation of reactive oxygen intermediates (31). CM-H₂DCFDA is a chloromethyl derivative of H₂DCFDA that exhibits a high retention in living cells. CM-H₂DCFDA passively diffuses into cells, where its acetate groups are cleaved by intracellular esterases, and its thiol-reactive chloromethyl group reacts with intracellular glutathione and other thiols. Subsequent oxidation yields a fluorescent adduct that is trapped inside the cell, thus facilitating long term studies (32).

In order to detect ROS formation at the single cell level, measurements were performed with hepatocytes cultured on glass coverslips (5 30 mm) using an inverted fluorescence microscope (Axiovert; Zeiss). For fluorescence recording, the coverslips were mounted with PBS at 37 °C, equilibrated with room atmosphere. Cells were excited at 488 nm at a rate of 2 Hz by a monochromator, and emission was measured at 515–565 nm using a CCD camera as provided by the QuantinC 2000-calcium imaging setup (VisiTech, Sunderland, UK). Emission intensity of unstimulated cells was set to 1, and increased emission intensity after the addition of CD95 ligand is expressed as relative increase of unstimulated cells was set to 1, and increased emission intensity after the addition of CD95 ligand is expressed as relative increase of unstimulated cells.

In order to detect ROS in a hepatocyte population, after a CM-H₂DCFDA loading period, cells were supplemented with culture medium. Cells were then exposed to CD95L for the indicated time period. Then cells were washed briefly using ice-cold PBS, and cells were lysed in 0.1% Triton X-100 (v/v) dissolved in aqua bidest. Lysates were incubated immediately (10 min) at 4 °C, 1 min, and fluorescence of the supernatant was measured at 515–565 nm using a luminescence spectrometer LS-5B (PerkinElmer Life Sciences) at 488-nm excitation wavelength.

Since the NADPH oxidase inhibitor apocynin itself was recently reported to induce ROS in vascular fibroblasts (33), the effect of apocynin on ROS generation in hepatocytes was measured. At the dose used in these studies (300 μmol/liter), apocynin by itself increased 2′,7′-dichlorodihydrofluorescein diacetate fluorescence negligibly from 1.00 to 1.015 ± 0.002 in hepatocytes (n = 10).

**Lipid Extraction and High Performance Thin Layer Chromatography—**Cells were harvested at the indicated time points by scraping the cells off of the plate on ice. Pellets were washed and sonicated. Quantification of lipids was done using Folch extraction (data not shown). As further shown in Fig. 1C, Nox 2 (gp91pol) protein was not only found in rat hepatocytes but also in rat hepatocytes and hepatic stellate cells. RAW mouse and rat peritoneal macrophages served as a positive control. Rat and human Nox 2 (gp91pol) protein is known to represent a de glycosylated protein with a molecular mass of approximately 55 kDa (37), whereas the human protein is highly glycosylated with a molecular mass of approximately 80–120 kDa (37). Such differences in electrophoretic mobility between murine and human Nox 2 (gp91pol) were also found (Fig. 1C). The presence of gp91pol and Nox 2 in 24-h cultured rat hepatocytes was also confirmed immunocytochemically (data not shown). In line with the immunocytochemical findings, subfractionation studies on rat hepatocytes revealed an almost exclusive presence of gp91pol expression in the cytosol (Fig. 1D). Only a small amount of gp91pol was detected in the membrane fraction, with the majority of the gp91pol protein being cytosolic (Fig. 1D). These findings differ from those reported for macrophages, which exhibit a predominant location of the NADPH oxidase gp91pol subunit at the plasma membrane, whereas the p47pol subunit is primarily cytosolic (38). However, in hepatocytes, cytosolic gp91pol was most likely contained in very light membranes, because centrifugation of the cytosolic preparation at 100,000 × g for 2 h resulted in the sedimentation of immunoreactive gp91pol, whereas the cytosolic marker enzyme GAPDH remained soluble (Fig. 1D). This finding indicates that...
hepatocytic gp91phox may be contained in membrane vesicles inside the cytosol, as one might expect for a transmembrane integral protein (15–18).

**Activation of NADPH Oxidase Isoforms by CD95L—**p47phox known as an adapter molecule, which is essential for activation of Nox 2 NADPH oxidase complex (39). p47phox activation was shown to involve serine phosphorylation, which is also required for activation of the NADPH oxidase complex in macrophages and neutrophils (22, 40). It is not yet established whether p47phox is also required for the in vivo activation of other Nox/Duox isoenzymes. As shown in Fig. 2A, CD95L induced a rapid Ser phosphorylation of p47phox in rat hepatocytes, which persisted for more than 60 min.

In order to address a potential role of p47phox and Nox/Duox enzymes for the generation of oxidative stress in response to CD95L, single cell fluorescence measurements were performed with DCFDA-loaded hepatocytes. DCFDA is frequently used as a probe for measurement of overall oxidative stress (31). Exposure of 24-h cultured rat hepatocytes to CD95L (100 ng/ml) induced within 1 min an oxidative stress response, as detected by DCFDA fluorescence (Fig. 2B, Table I). This CD95L-induced oxidative stress response was not only sensitive to N-acetylcysteine (1) but was also largely abolished by apocynin (Fig. 2B), diphenyleneiodonium, and neopterin (Table I) (i.e. known inhibitors of NADPH oxidase). These findings suggest a role of NADPH oxidase isoforms in triggering the CD95L-induced oxidative stress response. However, one has to keep in mind that these inhibitors may have unspecific side effects.

Therefore, the potential role of Nox/Duox enzymes in generating the rapid CD95L-induced oxidative stress response was further studied in 4-day cultured rat hepatocytes following transfection with nonsense or p47phox antisense oligonucleotides. 4 days after transfection with the antisense but not the nonsense oligonucleotides resulted in a strong inhibition of the p47phox protein (Fig. 3A). As shown in Fig. 3B, p47phox protein knockdown, but not transfection with the nonsense oligonucleotides resulted in a strong inhibition of the CD95L-induced increase of DCFDA fluorescence. These data suggest that Nox 2 and/or other p47phox-dependent NADPH oxidase isoforms are involved in the rapid oxidative stress response, which is triggered by CD95L.

**Mechanism of CD95L-induced Activation of NADPH Oxidase Isoforms—**Inhibitor studies were performed in order to gain insight into the upstream signaling events leading to CD95L-induced p47phox phosphorylation and ROS formation. As shown in Fig. 4A, CD95L-induced p47phox-Ser phosphorylation was sensitive to AY9944 and desipramine (i.e. inhibitors of sphingomyelinase) (41, 42), suggesting that sphingomyelinase activation is upstream of CD95L-induced p47phox phosphorylation. As shown by immunocytochemistry, acidic sphingomyelinase was not only found inside the hepatocytes in a presumably lysosomal compartment but also at the plasma membrane (Fig. 4B). In line with a CD95L-induced activation of sphingomyelinase, CD95L produced within 30 s a significant increase in

**Fig. 1. mRNA expression of NADPH oxidase isoforms in rat liver cells.** Hepatocytes (PC), Kupffer cells (KC), and hepatic stellate cells (HSC) were isolated as described under “Experimental Procedures.” Hepatocytes were cultured for 1 or 4 days (PC d1/d4), Kupffer cells were cultured for 2 days (KC), and hepatic stellate cells were kept for 1 or 14 days in culture (HSC d1/d14), respectively. Thereafter, cells were harvested, and mRNA expression of Nox 1–4, Duox 1 and 2, and the NADPH oxidase regulatory subunit p47phox was detected by RTPCR (A). mRNA isolated from fetal rat kidney served as a positive control for Nox 3 (19, 20). GAPDH expression served as a loading control (n = 3). All PCR products were confirmed by sequencing after isolation from agarose gels. B, the NADPH oxidase regulatory subunit p47phox was detected by Western blotting in rat hepatocytes. GAPDH expression served as a loading control (n = 3), C, gp91phox (i.e. Nox 2) was also detected in rat hepatocytes, Kupffer cells, and hepatic stellate cells. Rat peritoneal macrophages and the mouse RAW macrophages served as positive controls. In addition, human hepatoma cell line 7 (Huh7) and hepatoma cell line G2 (HepG2) were detected for gp91phox expression. Here, human monocytes served as a positive control. Whereas murine cells revealed a band at ~55 kDa, which probably reflects the unglycosylated gp91phox, human monocytes showed a smear at ~85–120 kDa reflecting the glycosylated protein, as reported previously (37). In contrast to rat hepatocytes, human hepatoma cells (i.e. Huh7 and HepG2) showed no gp91phox expression. D, plasma membrane and cytosolic fractions of PC were obtained as described under “Experimental Procedures” (i.e. by centrifugation at 40,000 × g for 35 min) and detected for gp91phox (Nox 2) and the regulatory subunit p47phox gp91phox (~55 kDa) revealed a predominant expression in the supernatant (cytosol) and only small amounts of gp91phox in the pellet (i.e. plasma membrane fraction). Higher molecular mass forms of gp91phox were not detectable. After ultracentrifugation (100,000 × g for 2 h) of this supernatant, gp91phox was found in the pellet, whereas p47phox and GAPDH were still found in the supernatant. These findings suggest that gp91phox but not p47phox is probably membrane-associated and contained in intracellular membrane vesicles. GAPDH and annexin II served as markers for the cytosolic and the plasma membrane fractions, respectively. GAPDH was not detectable in the membrane fraction, and annexin II was not detectable in the cytosolic fraction, indicating a high efficacy of separation.

**Fig. 2.** (A) Immunoblotting of p47phox in rat hepatocytes, Kupffer cells, and hepatic stellate cells. p47phox was detected by RT-PCR (A). (B) Activation of CD95L produced within 30 s a significant increase in...
ceramide levels, which lasted for more than 30 min (Fig. 4C). The CD95L (100 ng/ml)-induced increase in ceramide levels within 1 min was 1.87 ± 0.06-fold (n = 5) compared with unstimulated control, which was arbitrarily set to 1. When AY9944 (5 μmol/liter) or desipramine (5 μmol/liter) was preincubated for 30 min prior to CD95L (100 ng/ml) addition for 1 min, this increase of ceramide levels was largely abolished (Fig. 4D). These findings suggest the involvement of sphingomyelinase-derived ceramide in triggering CD95L-induced p47phox serine phosphorylation. In line with this, the addition of C6- and C16-ceramide but not of the inactive C6-dihydroceramide did induce p47phox Ser phosphorylation (Fig. 4A).

Because several protein kinases, among them some PKC isoforms were reported to induce serine phosphorylation of p47phox (40), the role of various PKC isoforms in triggering CD95L-induced p47phox serine phosphorylation was investigated. Rat hepatocytes are known to express the PKC isoforms α, βII, δ, ε, and ζ (43, 44). Conventional, Ca2+-dependent PKC isoforms α, β, and γ and the novel Ca2+-independent isoforms δ and ε are sensitive to inhibition by Go6850, whereas Go6976 and Ro-32-0432 exhibit more specificity on the classical PKC isoforms (45, 46). Neither Go6850, Go6976, nor Ro-32-0432 abolished p47phox pseudosubstrate phosphorylation (Fig. 4A).

Specific inhibition of PKCζ by a synthetic PKCζ-pseudosubstrate abolished p47phox phosphorylation in response to CD95L. Also chelerythrine, an unspecific PKC inhibitor with some effect on PKCδ and ε, blunted CD95L-induced p47phox phosphorylation. On the other hand, specific inhibitors of the PKC isoforms ε, η, and θ were ineffective (Fig. 4A). The inhibitory PKCζ substrate also blocked p47phox Ser phosphorylation in response to C6-ceramide, suggesting that PKCζ is involved in the signaling of ceramide toward p47phox (Fig. 4A) (48). In line with a CD95L-induced activation of

| 24-h cultured hepatocytes | Relative increase in CM-H2DCFDA fluorescence |
|--------------------------|---------------------------------------------|
| Control                  | Set as 1                                    |
| CD95L                    |                                            |
| + AY9944                 | 1.48 ± 0.06*                                |
| + Desipramine            | 1.05 ± 0.03*                                |
| + PKCζ inhibitor         | 1.07 ± 0.03*                                |
| + Chelerythrine          | 1.13 ± 0.05*                                |
| + Diphenylenedioinonid    | 1.17 ± 0.05*                                |
| + Apocynin               | 1.13 ± 0.05*                                |
| C6-dihydroceramide       | 1.22 ± 0.04*                                |
| C16-ceramide             | 1.22 ± 0.04*                                |
| PKCζ inhibitor           | 1.22 ± 0.04*                                |
| Chelerythrine            | 1.18 ± 0.05*                                |
| Apocynin                 | 1.23 ± 0.04*                                |
| Neopterin                | 1.24 ± 0.06*                                |
| C6-dihydroceramide       | 1.33 ± 0.06*                                |
| 96-h cultured hepatocytes|                                            |
| Relative increase in CM-H2DCFDA fluorescence |                                    |
| Control                  | Set as 1                                    |
| CD95L                    |                                            |
| + Nonsense oligonucleotides | 1.46 ± 0.12*                               |
| + p47phox-antisense oligonucleotides | 1.10 ± 0.04*                               |

* p < 0.05.
** p < 0.01.
*** p < 0.001.
**** p > 0.05, not significant.
PKCζ, this PKC isoform was translocated to the plasma membrane of hepatocytes in response to CD95L (Fig. 4E).

As shown in Fig. 5 and Table II, both, inhibition of sphingomyelinase by AY9944 or desipramine and inhibition of PKCζ by chelethryamine or the specific PKCζ substrate (i.e. inhibitors of CD95L-induced p47phox serine phosphorylation and of the early CD95L-induced ROS response) (Fig. 4A, Table II) blunted CD95L-induced Yes activation (see below) (Fig. 10). Further, as shown in Fig. 6F, Yes activation in response to CD95L was blunted in the presence of DPI, apocynin, and neopterin. These findings suggest that the early CD95L-induced ROS response occurs via NADPH oxidase activation and represents an upstream signal for CD95L-induced Yes activation.

Nox-dependent Oxidative Stress and JNK Activation in Response to CD95L—As shown recently (1), CD95L triggers within 5–10 min an activation of JNK. CD95L-induced JNK activation was insensitive to SU6656 (Fig. 7), PP-2, or herbimycin A (not shown), indicating that Yes, Fyn, or c-Src are not involved as upstream events. However, CD95L-induced JNK activation was largely inhibited in the presence of DPI, apocynin, and neopterin (Fig. 7) or after p47phox knockdown (see below) (Fig. 10). Likewise, inhibition of sphingomyelinase by AY9944, desipramine or of PKCζ by its inhibitory substrate or chelethryamine blunted CD95L-induced JNK activation (Fig. 7). These data suggest that not only CD95L-induced Yes activation but also JNK activation involves the action of reactive oxygen species generated by NADPH oxidase isoforms. The data further suggest that Yes is not upstream of CD95L-induced JNK activation, because the latter was not abolished after Yes inhibition by SU6656.

Yes Triggers EGFR Activation in Response to CD95L—Previous studies on ligand-independent activation of the CD95 system in rat hepatocytes by hydrophobic bile acids or hyperosmolarity revealed a Yes-dependent activation of the EGFR as another upstream event leading to CD95 activation (4, 5). Therefore, experiments were performed in order to study Yes/EGFR interactions in response to CD95L. As shown in coimmunoprecipitation studies, CD95L induced within 1 min an association between Yes and the EGFR (Fig. 8A), which gradually disappeared thereafter. No association between EGFR and Fyn or c-Src was found (data not shown). CD95L-induced Yes/EGFR association was inhibited by SU6656, indicating the requirement of active Yes for this association (Fig. 6F). In line with this, also NAC, DPI, apocynin, and neopterin, which inhibit CD95L-induced Yes activation, blunted Yes/EGFR asso-
exposed to CD95L (100 ng/ml). Ceramide was measured as described under “Experimental Procedures.” CD95L significantly increases ceramide.

In another set of experiments, chelerythrine (20 μmol/liter) or desipramine (5 μmol/liter) for 1 min. Then cells were stained for PKCβ membrane. Acidic sphingomyelinase expression was also detected by Western blotting in hepatocytes (PKCβ), and hepatic stellate conditions PKCβ shows an intracellular immunostaining, CD95L induced an enrichment of PKCβ membrane suggestive of a CD95L-induced PKCβ translocation to the plasma membrane. 24-h cultured rat hepatocytes were exposed to either control medium or CD95L (100 ng/ml) for 1 min. Then cells were stained for PKCβ as described under “Experimental Procedures.” Whereas under control conditions PKCβ mainly shows an intracellular immunostaining, CD95L induced an enrichment of PKCβ immunoreactivity at the plasma membrane suggestive of a CD95L-induced PKCβ membrane translocation (n = 3).

EGFR/CDC95 Interactions in Response to CD95L—CD95L-induced EGFR-tyrosine phosphorylation was sensitive to SU6656, NAC, DPI, apocynin, and neopterin but insensitive to PP-2, herbimycin, JNK inhibitor, or AG1478 (Fig. 6F) and was also inhibited by AY9944, desipramine, and the PKCβ substrate (Fig. 6, D and E). This inhibitor profile and the finding that Yes, but not Fyn or c-Src, rapidly associates with the EGFR in response to CD95L suggest that CD95L-induced EGFR activation is mediated by Yes, as it was shown recently for EGFR activation in response to hyperosmolality or proapoptotic bile acids (4, 5).

EGFR/CDC95 Interactions in Response to CD95L—CD95L, hyperosmolality, and proapoptotic bile salt were shown to induce an EGFR-dependent activation of the CD95 system through EGFR-catalyzed CD95L-tyrosine phosphorylation, which was identified as a crucial step for CD95 trafficking to the membrane, formation of the death-inducing signaling complex (DISC), and apoptosis induction (1, 2). These proapoptotic stimuli trigger within 30 min a JNK-dependent intracellular...
association of the EGFR and CD95, which is followed by an EGFR-catalyzed CD95-tyrosine phosphorylation (1, 2). Thereafter, translocation of the EGFR-CD95 protein complex to the plasma membrane and DISC formation occur and were assessed 2 h after the addition of the proapoptotic stimulus (1–3). As shown in Fig. 8A, about 30 min after CD95L addition, the Yes-EGFR complex starts to disappear, and EGFR increasingly associates with the CD95. No communoprecipitation of Yes and CD95 was detectable, indicating that EGFR dissociates from Yes prior to its association with CD95. In line with previous data (1, 2), EGFR/CD95 association was JNK inhibitor-sensitive and was followed by an EGFR-catalyzed CD95-tyrosine phosphorylation, which was sensitive to inhibition by AG1478 and genistein (Fig. 6F). This CD95-tyrosine phosphorylation was recently shown to be essential for CD95 translocation to the plasma membrane and recruitment of FADD and caspase 8 (i.e. formation of the DISC) (1–3). All of these events were strongly blunted by SU6656 but not by PP-2 (Fig. 6F). Also, inhibitors of NADPH oxidases (i.e. DPI, apocynin, and neopterin) (Fig. 6F) and inhibition of sphingomyelinase or PKCζ as upstream events in the CD95L-induced ROS formation blunted these responses (Fig. 6D). These inhibitors also strongly blunted the previously described (1) CD95 translocation to the plasma membrane in response to CD95L (Table III). A similar inhibitor profile of CD95L-induced membrane translocation as observed in rat hepatocytes was also found in Huh7 cells that were transfected with CD95-YFP (Table III). These data strongly support the view that CD95L-induced activation of NADPH oxidase isoenzymes is an important upstream event in the activation of the CD95 system including DISC formation.

In order to demonstrate the requirement of sphingomyelinase, PKCζ, and NADPH oxidases for the CD95L-induced EGFR/CD95 interactions in the living cell, FRET experiments were performed in Huh7 cells (26). For this purpose, Huh7 cells, which exhibit almost no endogenous CD95 expression (55), were cotransfected with EGFR-YFP and CD95-CFP, as described recently (3). In line with previous data (3) and as shown in Fig. 9, CD95L induced within 30 min a cytosolic FRET signal (Fig. 9A), suggestive of intracellular EGFR/CD95 association. After 2 h, the EGFR-CD95 complex was targeted to the plasma membrane (Fig. 9B), as evidenced by a strong FRET signal in the plasma membrane. Prevention of CD95L-induced NADPH oxidase activation by apocynin, sphingomyelinase inhibition (AY9944), or PKCζ inhibition by its inhibitory substrate prevented both cytosolic EGFR/CD95 association and targeting of the protein complex to the plasma membrane (Fig. 9, A and B, and Table IV).

**Role of NADPH Oxidases, Yes, and EGFR in CD95L-induced Hepatocyte Apoptosis**—In order to substantiate the roles of NADPH oxidases, Yes, and the EGFR in CD95L-induced hepatocyte apoptosis, knockdown experiments after transfection with respective nonsense and antisense oligonucleotides were performed. As shown in Figs. 3A and 10, after 4 days of culture, a significant down-regulation of p47^phox, Yes, and EGFR was obtained, when rat hepatocytes were transfected with corresponding antisense oligonucleotides. As shown in Fig. 10, p47^phox knockdown strongly inhibited CD95L-induced Yes, JNK, and EGFR activation as well as EGFR/CD95 association, CD95-tyrosine phosphorylation, CD95 translocation to the plasma membrane, and DISC formation. Similar results were obtained after Yes knockdown except for a preserved JNK activation and EGFR/CD95 association in response to CD95L (Fig. 10). This is an expected finding, because Yes knockdown does not affect the CD95L-induced oxidative stress response and, accordingly, JNK activation. The latter was shown to be required for EGFR/CD95 association, which occurs irrespective of the EGFR activation status (1, 2). Also, EGFR knockdown resulted in an inhibition of CD95L-induced oxidative stress response and, accordingly, JNK activation. This was shown to be required for EGFR/CD95 association, which occurs irrespective of the EGFR activation status (1, 2).

**DISCUSSION**

**CD95L-induced Early Oxidative Stress Response**—In rat hepatocytes, proapoptotic stimuli, such as CD95L (1), hydrophobic bile acids (2), or hyperosmotic cell shrinkage (1) trigger a rapid oxidative stress response, as assessed by an increase in 2',7'-dichlorodihydrofluorescein diacetate fluorescence. Inhibition of this oxidative stress response by antioxidants was accompanied by an inhibition of hepatocyte apoptosis (1, 2, 5), suggesting its requirement for CD95-dependent apoptosis induction. Mitochondria have repeatedly been shown to be a source of oxidative stress in response to proapoptotic stimuli (56, 57), including ligands of the tumor necrosis factor receptor family (11, 58). However, as shown in the present study, the almost immediate CD95L-induced ROS response apparently
FIG. 6. CD95 ligand-induced activation of Src family kinases and activation of the CD95 system in rat hepatocytes. Hepatocytes were cultured for 24 h and then exposed to CD95L (100 ng/ml) for the given time periods. When indicated, AY9944 (5 μmol/liter), desipramine (5 μmol/liter), PKCζ inhibitor (100 μmol/liter) or chelerythrine (20 μmol/liter), diphenyleneiodonium (10 μmol/liter), apocynin (300 μmol/liter), d(+)-neopterin (100 μmol/liter), NAC (30 mmol/liter), SU6656 (10 μmol/liter), PP-2 (10 μmol/liter), herbimycin A (1 μmol/liter), t-JNKI1 (5 μmol/liter), genistein (100 μmol/liter), AG1478 (5 μmol/liter) were preincubated for 30 min. The Src family kinases Yes and Fyn were immunoprecipitated (IP) as described under "Experimental Procedures," and their activating phosphorylation at position Tyr418 was detected by Western blotting. c-Src phosphorylation at position Tyr418 was detected by Western blotting using phosphospecific antibodies. A and B, CD95L induced within 1 min activation of Yes, which was sensitive to SU6656 and NAC but not to PP-2. Fyn and Src phosphorylation was sensitive to SU6656 and PP-2, whereas NAC was ineffective (n = 3; A). Densitometric analysis of Yes, Fyn, and Src phosphorylation at position Tyr418 showed a significant inhibition of CD95L-induced Yes activation by NAC (*, p < 0.05) but not of CD95L-induced Fyn and Src activation, respectively (p > 0.05; B). C, concentration dependence of CD95L- or H$_2$O$_2$-induced Yes-Tyr418 phosphorylation as assessed 1 min after CD95L or H$_2$O$_2$ addition, respectively (n = 3). CD95L-induced Yes phosphorylation exhibited a similar concentration dependence as observed for CD95L-induced p47$^{phox}$-serine phosphorylation (n = 3). D–F, Yes, EGFR, and CD95 were immunoprecipitated as described under "Experimental Procedures" and analyzed by Western blotting. Activating Yes-Tyr418 phosphorylation, YES/EGFR association, and EGFR-tyrosine phosphorylation (EGFR-Tyr-P) were detected 1 min after CD95L addition; EGFR/CD95 association and CD95-tyrosine phosphorylation (CD95-Tyr-P) were detected after 60 min of CD95L exposure; and caspase 8/CD95 and FADD/CD95 association were detected 3 h after CD95L addition. Total Yes, EGFR, and CD95 served as respective loading controls. These time points were chosen based on time course studies in previous work on CD95 activation by hyperosmolarity (1, 4) or hydrophobic bile salts (2, 5). D, CD95L induced within 1 min a Yes activation and Yes/EGFR association followed by a Yes-mediated
involves the action of NADPH oxidase isoforms, whose presence in rat hepatocytes could be shown at the level of mRNA expression and for Nox 2 and the regulatory subunit p47^phox also at the protein level. Interestingly, Nox-2 and p47^phox protein was located in 24-h cultured rat hepatocytes mainly in the cytosol. This distribution is different from that described in macrophages, which exhibit a predominant plasma membrane localization of gp91^phox (Nox 2) (38).

CD95L-induced ROS formation via Nox/Duox enzymes was evidenced by a rapid Ser phosphorylation of the regulatory subunit p47^phox in response to CD95 ligand and an inhibition of the CD95L-induced ROS response by inhibitors of NADPH oxidases and after knockdown of p47^phox. DPI sensitivity of CD95 (Fas) ligation-induced ROS formation was also described in Jurkat cells (59). The present findings do not rule out a contribution of mitochondria to CD95L-induced ROS generation; however, our data suggest that the initial trigger for CD95L-induced ROS formation are NADPH oxidases, whereas mitochondrial ROS generation may be a downstream consequence of subsequent CD95 activation. If this view is correct, mitochondria may be seen as an amplifier of the initial ROS response triggered by NADPH oxidase activation. Which Nox/Duox isoform(s) is responsible for the CD95L-induced ROS response in hepatocytes is not settled. However, an unequivocal requirement of p47^phox for Nox 2 (gp91^phox) activation was demonstrated (16), whereas it is unclear whether other isoforms are also regulated in vivo by p47^phox. Thus, Nox 2 is a likely candidate for the CD95L-induced ROS response in hepatocytes; however, a contribution of other Nox/Duox isoforms is not ruled out.

In contrast to 24-h cultured rat hepatocytes, Nox 2 protein and mRNA were not expressed by the human hepatoma cell line HuH7, whereas mRNAs for p47^phox as well as Nox 1, 3, and 4 and Duox 1 and 2 were detectable. As in rat hepatocytes, also in CD95-YFP-transfected HuH7 cells, apocynin, the PKC^pseudosubstrate, and AY9944 inhibited CD95L-induced CD95 translocation to the plasma membrane and apoptosis induction. These findings suggest that in HuH7 cells NADPH oxidase isoforms distinct from Nox 2 may be involved in the induction of apoptosis by CD95L.

The inhibitory action of apocynin on CD95L-induced ROS formation in rat hepatocytes deserves some comment. Previous studies have shown that inhibition of NADPH oxidase by apocynin is not mediated by apocynin itself but by a compound derived from apocynin in a peroxidase-dependent way (60). Due to this mechanism, apocynin was shown to inhibit NADPH oxidase in phagocytic cells, whereas in some (peroxidase-deficient) nonphagocytic cells, such as fibroblasts, apocynin stimulates rather than inhibits ROS formation (33). However, the inhibitory effect of apocynin was restored following treatment with H_2O_2 and horseradish peroxidase (33). Hepatocytes are known to contain high activities of peroxidases (61, 62) and to exhibit endogenous H_2O_2 formation (61, 63), which are prerequisites for the inhibitory action of apocynin on NADPH oxidases. This may explain why in rat hepatocytes after a 30-min preincubation with apocynin, CD95L-induced ROS formation via NADPH oxidase is inhibited.

**Mechanism of CD95L-induced NADPH Oxidase Activation**—Sphingomyelinase, ceramide, and PKC\_\textsuperscript{\textgamma} were identified as up-

EGFR-tyrosine phosphorylation, which was sensitive to inhibition of sphingomyelinase and PKC\_\textsuperscript{\textgamma}. Also, EGFR/CD95 association and CD95-tyrosine phosphorylation as well as DISC formation were largely prevented by inhibitors of sphingomyelinase and PKC\_\textsuperscript{\textgamma} (\(\alpha = 3\)). F, densitometric analysis of CD95L-induced Yes-Tyr\textsuperscript{418} phosphorylation, Yes/EGFR association, and subsequent EGFR-tyrosine phosphorylation revealed a significant effect of inhibition of sphingomyelinases (AY9944 and desipramine) and PKC\_\textsuperscript{\textgamma} (PKC\_\textsuperscript{\textgamma} inhibitor and chelerythrine) (*, \(p < 0.05\)). F, also inhibitors of NADPH oxidase (DPI, apocynin, and neopterin) and Yes (SU6656) as well as NAC blunted CD95L-induced Yes activation, Yes/EGFR association, and EGFR-tyrosine phosphorylation (\(\alpha = 3\)). All maneuvers that prevented Yes activation also prevented EGFR-tyrosine kinase-mediated CD95-tyrosine phosphorylation and subsequent recruitment of FADD and caspase 8 to CD95 (DISC formation) (\(\alpha = 3\)).
stream events of CD95L-induced NADPH oxidase activation. In line with this, both CD95L-induced p47<sup>phox</sup> phosphorylation and the ROS response were sensitive to inhibitors of sphingomyelinase or PKC<sub>H9256</sub>. CD95L rapidly increased the formation of ceramide in a sphingomyelinase inhibitor-sensitive way. Although one has to keep in mind that some of these inhibitors may exert unspecific side effects, the findings indicate that sphingomyelinase-mediated ceramide formation may trigger PKC<sub>H9256</sub> activation as an upstream event in CD95L-induced NADPH oxidase activation. It is interesting to note that p47<sup>phox</sup> was recently shown to be a direct phosphorylation target of PKC<sub>H9256</sub> (48), and this PKC isoform participates in neutrophil respiratory burst (39, 48). Multiple roles of PKC<sub>H9256</sub>, a member of the atypical PKC group, in cell signaling have been described (for a review, see Ref. 64), and evidence has been given that ceramide can bind to and activate PKC<sub>H9256</sub> (for a review, see Ref. 65).

A role of ceramide formation in apoptosis has repeatedly been discussed (for a review, see Ref. 57). Evidence has been presented in leukemia cell lines and lymphocytes for an involvement of acidic sphingomyelinase in the apoptotic signaling through CD95 (66) and for a requirement of ceramide-mediated clustering for CD95-DISC formation (67). Further, ceramide was reported to overcome the apoptosis resistance of acidic sphingomyelinase-deficient mouse hepatocytes and lymphocytes (68, 69) and was suggested in various cell types to mediate at least in part its apoptotic effect through activation of the JNK pathway (65, 70–72). A role of ceramide in activating JNK in response to CD95L is suggested by the finding that CD95L-induced JNK activation was sensitive to inhibition of sphingomyelinase by AY9944 and desipramine. However, CD95L-induced JNK activation was also sensitive to inhibitors of PKC<sub>H9256</sub> and NADPH oxidases and NAC but was insensitive to Yes inhibition by SU6656. These data suggest that CD95L-induced early ROS formation may trigger JNK and Yes activation in parallel. The mechanisms underlying early CD95L-induced sphingomyelinase activation remain speculative, as are the sphingomyelinase isoenzymes, which are involved in this response. Potential activation mechanisms have been reviewed recently (65, 73).

CD95L-induced Activation of Src Family Kinases—CD95L triggered a rapid activation of the Src kinase family member Yes, which apparently requires NADPH oxidase-mediated ox-
Top, hepatocytes were cultured for 24 h and then exposed to CD95L (100 ng/ml) for 3 h in order to detect CD95 translocation to the plasma membrane by immunostaining or for 12 h in order to determine hepatocyte apoptosis using the TUNEL assay. When indicated, AY9944 (5 μmol/liter), desipramine (5 μmol/liter), PKCζ inhibitor (100 μmol/liter), chelerythrine (20 μmol/liter), NAC (30 mmol/liter), diphenylethionidium (10 μmol/liter), apocynin (300 μmol/liter), d(-)-neopterin (100 μmol/liter), SU6656 (10 μmol/liter), or PP-2 (10 μmol/liter) was added 30 min prior to CD95L addition. In line with previous data (1), CD95L induced CD95 translocation to the plasma membrane, which was sensitive to inhibitors of sphingomyelinase, PKCζ, NADPH oxidase, and SU6656 but not to PP-2. A similar inhibitor profile was found for CD95L-induced hepatocyte apoptosis. Data are given as means ± S.E. (n = 3 different preparations). Middle, cultured HuH7 cells were transfected with CD95-YFP as described under 'Experimental Procedures' and then exposed to CD95L (100 ng/ml) for 3 h in order to detect CD95-YFP translocation to the plasma membrane or for 12 h in order to determine HuH7 cell apoptosis using the TUNEL assay. When indicated, AY9944 (5 μmol/liter), PKCζ inhibitor (100 μmol/liter), or apocynin (300 μmol/liter) was added 30 min prior to CD95L addition. In line with previous data (3), CD95L induced CD95 translocation to the plasma membrane, which was sensitive to inhibitors of sphingomyelinase, PKCζ, and NADPH oxidase. A similar inhibitor profile was found for CD95L-induced HuH7 cell apoptosis. Data are given as means ± S.E. (n = 3 different transfections). Bottom, in another set of experiments, hepatocytes were cultured for 96 h with either nonsense oligonucleotides or p47phox-, Yes-, or EGFR-antisense nucleotides as described under 'Experimental Procedures' and stained immunocytochemically for CD95 membrane translocation 3 h after CD95L (100 ng/ml) addition. The percentage of apoptotic cells was detected using the TUNEL assay after 12 h of exposure to CD95L (100 ng/ml). In nonsense oligonucleotide-treated hepatocytes, CD95L induced CD95 membrane translocation and apoptosis compared to untreated control, which was inhibited after p47phox, Yes, and EGFR knockdown. Data are given as means ± S.E. (n = 3 different preparations).

### Table III

**Inhibition of CD95 ligand-induced CD95 membrane translocation and apoptosis by inhibition of sphingomyelinase, PKCζ, NADPH oxidase, Yes, and protein knockdown of p47phox, Yes, or EGFR**

| 24-h cultured rat hepatocytes | Cells with CD95 membrane staining | Cells with positive TUNEL staining |
|-----------------------------|----------------------------------|----------------------------------|
| Control                     | 0.3 ± 0.1                        | 0.6 ± 0.3                        |
| CD95L                       | 11.4 ± 0.9                       | 85.1 ± 4.6                       |
| + AT9944                    | 3.6 ± 0.8                        | 43.8 ± 5.5                       |
| + Desipramine               | 3.7 ± 0.9                        | 47.9 ± 7.1                       |
| + PKCζ inhibitor            | 3.1 ± 0.5                        | 41.1 ± 7.0                       |
| + Chelerythrine             | 3.5 ± 0.8                        | 48.1 ± 8.2                       |
| + N-Acetylcyesteine         | 5.7 ± 0.6                        | 41.4 ± 3.9                       |
| + Diphenylethionidium       | 6.5 ± 0.8                        | 46.6 ± 5.0                       |
| + Apocynin                  | 5.9 ± 0.7                        | 43.2 ± 5.1                       |
| + Neopterin                 | 6.9 ± 0.9                        | 47.2 ± 4.4                       |
| + SU6656                    | 4.3 ± 0.6                        | 39.5 ± 4.5                       |
| + PP-2                      | 10.7 ± 0.9                       | 78.1 ± 4.7                       |

| 96-h cultured rat hepatocytes | Cells with CD95 membrane staining | Cells with positive TUNEL staining |
|-----------------------------|----------------------------------|----------------------------------|
| Control                     | 1.2 ± 0.4                        | 2.9 ± 0.6                        |
| CD95L                       | 22.9 ± 1.3                       | 41.6 ± 4.0                       |
| + AT9944                    | 7.6 ± 0.9                        | 14.3 ± 2.3                       |
| + PKCζ inhibitor            | 5.7 ± 0.9                        | 11.8 ± 1.4                       |
| + Apocynin                  | 6.6 ± 0.8                        | 12.9 ± 1.8                       |

| 24-h cultured rat hepatocytes | Cells with CD95-YFP membrane staining | Cells with positive TUNEL staining |
|-----------------------------|--------------------------------------|----------------------------------|
| Control                     | 11.1 ± 1.1^b_1^                       | 80.9 ± 4.3                       |
| CD95L                       | 11.1 ± 1.1^a                         | 80.9 ± 4.3                       |
| + p47phox-antisense oligonucleotides | 0.5 ± 0.2                              | 1.9 ± 0.3                       |
| Control                     | 6.5 ± 0.8^b                       | 51.9 ± 4.4                       |
| CD95L                       | 11.3 ± 0.8^a                       | 81.5 ± 4.7                       |
| + p47phox-antisense oligonucleotides | 0.2 ± 0.1                              | 2.1 ± 0.3                       |
| Control                     | 5.2 ± 0.8^b                       | 48.5 ± 4.1                       |
| CD95L                       | 10.8 ± 1.0^a                       | 79.2 ± 4.6^c                      |
| + EGFR-antisense oligonucleotides | 0.2 ± 0.1                              | 0.9 ± 0.2                       |
| Control                     | 4.5 ± 0.6^b                       | 40.8 ± 3.6^b                      |

a *p < 0.05.

b *p < 0.05.

c *p > 0.05; not significant.

Idiopathic stress as an upstream event. An antioxidant-sensitive Yes activation was recently also found in response to other proapoptotic stimuli such as hyperosmotic hepatocyte shrinkage (4) or hydrophobic bile acids (5). In line with these latter studies, also CD95L-induced CD95 activation and DISC formation were dependent upon this Yes signal. Yes knockdown inhibited CD95L-induced CD95 activation, DISC formation, and apoptosis induction. In addition to Yes, CD95L also induced after some delay an activating phosphorylation of Fyn and c-Src, which, however, was not antioxidant-sensitive, indicating that the ROS signal is required for Yes but not for Fyn and c-Src activation. Mechanisms and significance of CD95L-induced Fyn and c-Src activation are a matter of speculation; however, in contrast to Yes inhibition by SU4466, Fyn and c-Src inhibition by PP-2 had no effect on CD95L-induced CD95 activation and apoptosis induction. Thus, Fyn and c-Src may not play a major role in apoptosis induction. In line with this, hydrophobic bile acids, such as taurolithocholate-3-sulfate, activated Yes and the CD95 system and induced apoptosis but had no effect on Fyn and c-Src phosphorylation (5).

The mechanisms underlying Yes activation in response to CD95L-induced oxidative stress or externally added H₂O₂ are
unknown; however, oxidative stress was shown to inhibit protein phosphatases (74, 75), which in turn could trigger inhibition of Yes dephosphorylation. In line with such a mechanism, inhibition of protein phosphatases by vanadate was shown to activate dephosphorylation. In line with such a mechanism, inhibition of protein phosphatases (74, 75), which in turn could trigger inhibition of Yes in rat hepatocytes (5).

Role of the EGFR in CD95L-induced Apoptosis—Activated

**A** EGFR-CFP | FRET | CD95-YFP
---|---|---
Control | 255 | 0
CD95L (30 min) + AY9944 | 255 | 0
AP35 + Apocynin | 255 | 0

Table IV

Inhibition of CD95 ligand-induced EGFR-CFP/CD95-YFP association and subsequent membrane translocation of the EGFR protein complex by inhibition of sphingomyelinase, PKCζ, or NADPH oxidase in Huh7 hepatoma cells

Huh7 hepatoma cells were cotransfected with EGFR-CFP and CD95-YFP and then exposed to CD95L (100 ng/ml) for 0 min (i.e. immediately before CD95L addition), 30 min, or 120 min, respectively. When indicated, AY9944 (5 μmol/liter), PKCζ inhibitor (100 μmol/liter), or apocynin (300 μmol/liter) were added 30 min prior to CD95L addition. About 49 ± 5% (n = 3) of the cells expressed the transfected EGFR-CFP and CD95-YFP constructs. FRET pictures were taken as described under “Experimental Procedures” and then normalised with respect to FRET efficiencies (scale indicates FRET efficiency from blue/0 to red/255), as described recently (3). A, CD95L induced within 30 min an intracellular FRET signal, indicating an intracellular EGFR-CFP/CD95-YFP association (n = 5). Inhibition of sphingomyelinase, PKCζ, or NADPH oxidase by AY9944, PKCζ inhibitor, or apocynin, respectively, prevented the intracellular EGFR-CFP/CD95-YFP association (n = 3). B, after 120 min of CD95L exposure, the EGFR-CFP/CD95-YFP protein complex was translocated to the plasma membrane as indicated by the FRET signal (n = 5), which was again sensitive to inhibition of sphingomyelinase, PKCζ, or NADPH oxidase, respectively (n = 3).

Yes rapidly associates with the EGFR and triggers its phosphorylation at tyrosine residues Tyr845 and Tyr1173. EGFR-Tyr845 is a known Src target (52), whereas Tyr1173 reflects an activating autophosphorylation site of the EGFR (53). Yes inhibition by SU6656 abolishes EGFR phosphorylation at both sites, whereas AG1478 inhibits EGFR-Tyr1173 phosphorylation.
Tyr1045 phosphorylation may in part explain why EGFR is not phosphorylated (3). All maneuvers that prevented activating EGFR-tyrosine phosphorylation were detected by Western blotting (n = 4). Yes and EGFR were immunoprecipitated as described under “Experimental Procedures.” The immunoprecipitated Yes and EGFR samples were then detected for activating phosphorylation by Src family-Tyr118 (Yes-Y118-P) or tyrosine phosphorylation (EGFR-Tyr-P) (n = 4). GAPDH, total Yes, total EGFR, and total JNK-1 served as loading controls. Representative Western blotting of four independent experiments are shown along with the respective loading controls. p47phox and Yes knockdown largely prevent CD95L-induced Yes and EGFR phosphorylation (stimulation for 1 min), whereas JNK activation (stimulation for 30 min) in response to CD95L, was affected by p47phox knockdown only. These parameters were not affected after EGFR knockdown. CD95 was immunoprecipitated as described under “Experimental Procedures.” CD95 samples were then detected for EGFR association, for tyrosine phosphorylation (CD95-Tyr-P), and for FADD and caspase 8 (Casp 8) association by Western blotting (n = 4). Total CD95 served as loading control. The total CD95 amount was also detected in membrane and cytosolic fractions obtained by ultracentrifugation as recently described (1, 2) and in studies on mutated CD95 (3). Huh7 cells transfected with receptors with Tyr/Phe exchanges in positions 232 and 291 (i.e. in the death domain of the CD95) are resistant toward CD95L-induced activation and apoptosis induction. These findings indicate that CD95L-signaling through NADPH oxidases is a crucial step in apoptosis induction. Fig. 11 summarizes our current view with respect to CD95 ligand-induced activation of the CD95 system. CD95L addition rapidly activates sphingomyelinase and ceramide formation, which in turn activates PKCζ. The latter leads to an activating serine phosphorylation of the NADPH oxidase regulatory subunit p47phox, which is followed by generation of ROS. ROS formation triggers an activation of the Src family kinase Yes, which then associates with and activates the EGFR. EGFR then dissociates from Yes and associates with CD95 in a JNK-dependent way, which leads to EGFR-tyrosine kinase-mediated CD95-Tyr95 phosphorylation, which is a prerequisite for microtubule-dependent translocation of CD95 to the plasma membrane and subsequent FADD and caspase 8 recruitment (i.e. DISC formation) (3).

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Involvement of NADPH Oxidase Isoforms and Src Family Kinases in CD95-dependent Hepatocyte Apoptosis
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