The tumor necrosis factor (TNF), Fas, and TNF-related apoptosis-inducing ligand (TRAIL) receptors (R) are highly specific physiological mediators of apoptotic signaling. We observed earlier that a number of FasR-insensitive cell lines could redirect the proapoptotic signal to an anti-apoptotic ERK1/2 signal resulting in inhibition of caspase activation. Here we determine that similar mechanisms are operational in regulating the apoptotic signaling of other death receptors. Activation of the FasR, TNF-R1, and TRAIL-R, respectively, rapidly induced ERK1/2.5 activation, an event independent from caspase activity. Whereas inhibition of the death receptor-mediated ERK1/2 activation was sufficient to sensitize the cells to apoptotic signaling from FasR and TRAIL-R, cells were still protected from apoptotic TNF-R1 signaling. This protection of the cells sensitized with cycloheximide, which is sufficient to sensitize the cells also to apoptosis by TNF-R1 stimulation, we noticed that adenovirus-mediated expression of constitutively active MKK1 could rescue the cells from apoptosis induced by the respective receptors by preventing caspase-8 activation. Taken together, our results show that ERK1/2 has a dominant protecting effect over apoptotic signaling from the death receptor. This protection, which is independent of newly synthesized proteins, acts in all cases by suppressing activation of the caspase effector machinery.

Programmed cell death or apoptosis is a self-destruction process implanted in most cells, ready to be activated to eliminate unwanted cells (1). Various activators and inhibitors, however, strictly regulate this elimination mechanism in response to external and internal signals. Proapoptotic signals may be transduced through a subset of cytokine receptors of the tumor necrosis factor (TNF) family, termed death receptors (DRs). Members of the TNF receptor family are characterized by similarly extracellular domains containing cysteine-rich repeats (2). The DRs also share a common intracellular domain, the death domain (DD), which confers to them the ability to induce apoptosis. By way of DD interaction, proteins of the death-inducing signaling complex (DISC) will be recruited to the receptor, and the apoptotic machinery will be activated. In parallel to this, other adaptor molecules may bind to the complex and modulate the response, some of them inducing survival. The number of known DRs has been growing since the first one was discovered, and it seems that additional receptors are yet to be discovered (for reviews, see Refs. 3–5). The Fas receptor (FasR) or CD95/CD95-APO-1, TNF receptor 1 (TNF-R1) and TRAIL receptors 1 and 2 (TRAIL-R1 or DR4, TRAIL-R2 or DR5) are members of this family of proteins (6–8). Although the four receptors share some common features in their structures, they also have specific characteristics. The FasR binds the adaptor protein FADD (9), which in turn recruits and activates procaspase-8 (7, 10). TNF-R1, however, does not bind FADD directly, but TRADD has to be engaged before FADD (11) and procaspase-8 (12) can be recruited to the receptor. Defining the components of the TRAIL-R DISC is still a controversial matter. Both caspase-8 and caspase-10 have been implicated as crucial mediators of TRAIL-induced apoptotic signaling, and both caspases have been identified as part of the TRAIL-R DISC (13–15). It has been proposed that TRAIL would induce apoptosis through FADD-dependent and -independent pathways (10, 11). However, recent studies suggest that both TRAIL-R1 and TRAIL-R2 recruit FADD and caspase-8, although TRAIL-R1 could still induce FADD-independent apoptosis in some situations (14, 15, 18). In all cases, recruitment and activation of caspase-8 leads to induction of effector caspases and ultimately to apoptosis (19).

Besides the FADD/caspase-8 signaling cascade, a number of other signaling pathways are activated by the DRs, most likely involving adaptor/regulator proteins specific to each receptor.

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1 The abbreviations used are: TNF, tumor necrosis factor; MAPK, mitogen-activated protein kinase; ERK1/2, extracellular-regulated kinase 1 and 2; MKK1, mitogen-activated protein kinase kinase 1; CA, constitutively active; HA, hemagglutinin; TRAIL, TNF-related apoptosis-inducing ligand; DR, death receptor; DISC, death inducing signaling complex; DD, death domain; CHX, cycloheximide; PI, propidium iodide; PBS, phosphate-buffered saline; X-gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; Z-VAD-fmk, benzoyloxycarbonyl-VAD-fluoromethylketone; FADD, Fas-associated death domain protein; JNK, c-Jun N-terminal kinase; TRADD, TNFR1-associated death domain protein.
Especially the TNF-R has been implied to have several important signaling functions apart from apoptotic signaling. TRAF2 and RIP, the latter which was first identified as a component of the Fas DISC, have been shown to bind to TRADD and are thus recruited to the TNF-R, both of them contributing to JNK and NF-kB activation (11, 20). Likewise, new signaling functions are emerging for the FasR. Among the molecules involved in Fas-mediated signaling, RIP (21), Daxx (22, 23), and FAP-1 (24, 25) have been shown to bind to the FasR, modulating its signal. Some inhibitor proteins act by mimicking one or another protein of the apoptotic cascade, diverting it into inactivation. Among them, FLIP, a caspase-8-like protein lacking proteolytic activity has been shown to block caspase-8 activation (26, 27). Members of the mitogen-activated protein kinase (MAPK) family have also been shown to be involved in the signaling downstream of the Fas or TNF receptors (28–31). Particularly, the MAPK ERK1/2, which is known to induce cell growth and differentiation, has been shown to promote survival in a number of situations (32, 33). It has also been suggested that TRAIL-R could, with the intermediary of FADD, recruit TRADD and activate NF-kB in this way (17). However, TRADD and RIP were reported absent from the TRAIL-induced DISC in vivo (14), although it could exist in different cell lines than the ones investigated.

We have previously shown that ERK1/2 activation is able to suppress Fas-induced apoptosis in activated T-cells (34, 35). Therefore, we wanted to examine whether the same protective effect could be seen in cancer cells. As members of the TNF receptor family are involved in removal of tumor cells by the immune system, development of resistance to such killing would impair the defense mechanism. In this context, we recently demonstrated that ERK1/2 has a role in rendering cells insensitive to FasR killing, as inhibition of ERK1/2 activation sensitizes HeLa cells to FasR-mediated apoptosis (36). Since HeLa cells also express TNF and TRAIL receptors (37), we wanted to investigate in this cell model whether ERK1/2 could protect against apoptosis induced through these other DRs in the same way as in Fas signaling, especially because the apoptotic signaling pathways induced by the three cytokines present common features in term of their DISC composition. In this study we show that inhibition of ERK1/2 is able to enhance the sensitivity of HeLa cells to TRAIL-R1, as well as to Fas, whereas TNF treated cells remain insensitive. The insensitivity in the case of the TNF-R seems to be caused by the additional protective effect could be detected in the degree of ERK1/2 activation as well as anti-apoptotic effect on the different DRs.

RESULTS

Inhibition of MAPK kinase 1 (MKK1) Sensitizes HeLa Cells to Fas and TRAIL-mediated Apoptosis but Not TNF—We compared the kinetics of apoptosis induction by Fas, TNF, or TRAIL receptor, after sensitization with either the expression of constitutively active CHX or the MKK1 inhibitor PD98059. In the absence of any specific treatment, HeLa cells were resistant to FasR and TNF-R1 stimulation, but showed some sensitivity to TRAIL-R-mediated apoptosis (Fig. 1), although to a lesser degree than many other tested TRAIL-responsive cell lines (data not shown). As previously shown, HeLa cells were markedly sensitized to

MATERIALS AND METHODS

Cell Culture and Reagents—HeLa cells were cultured in Dulbecco’s Modified Eagle’s Medium (Sigma) supplemented with 10% inactivated fetal calf serum, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator with 5% CO2 in air at 37 °C. HeLa cells were incubated with an agonistic anti-human FasR mAb, or 20 ng/ml in 0.1% sodium citrate plus 0.1% Triton X-100. The cells were incubated at 4 °C overnight before analyzing on a FACSscan flow cytometer (Becton Dickinson). The subdiploid peak was considered to be apoptotic cells as previously described (42). For caspase assay, whole cell extracts were made by freeze-thaw cycles in resuspension buffer (150 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4) with 1 mM phenylmethylsulfonyl fluoride. 1 μg each of lactic acid dehydrogenase (LDH) and β-galactosidase (β-gal) were recovered by centrifugation at 4 °C. Whole cell extract (15 g of protein as determined by Bradford assay

Electrophoretic Mobility Shift Assay—Cells were harvested in cold PBS/EDTA, lysed by freeze-thaw in buffer C (25% glycerol, 0.42% NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 20 mM HEPES) containing phenylmethylsulfonyl fluoride and dithiothreitol (0.5 mM each), and supernatant was recovered by centrifugation at 4 °C. Whole cell extract (15 μg) was incubated with a 32P-labeled oligonucleotide reproducing the consensus NF-kB binding sequence. The protein-DNA complexes were then resolved on 4% polyacrylamide native gel electrophoresis.

Inhibition of MAPK kinase 1 (MKK1) Sensitizes HeLa Cells to Fas and TRAIL-mediated Apoptosis but Not TNF—We compared the kinetics of apoptosis induction by Fas, TNF, or TRAIL receptor, after sensitization with either the expression of constitutively active CHX or the MKK1 inhibitor PD98059. In the absence of any specific treatment, HeLa cells were resistant to FasR and TNF-R1 stimulation, but showed some sensitivity to TRAIL-R-mediated apoptosis (Fig. 1), although to a lesser degree than many other tested TRAIL-responsive cell lines (data not shown). As previously shown, HeLa cells were markedly sensitized to

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Fas-induced apoptosis by cotreatment with PD98059. Cotreatment with PD98059 rendered HeLa cells even more sensitive to TRAIL-R-mediated apoptosis, showing that ERK1/2 signaling has a role in the protection against both TRAIL-R- and FasR-induced apoptosis. In contrast, cells remained resistant to TNFα-induced apoptosis after PD98059-mediated inhibition of ERK1/2 activation, revealing that the TNF-R has additional survival mechanisms in action. To confirm the presence of functional DRs, we treated the cells with CHX, as many cells resistant to DR stimulation have been found to be sensitized by treatment with CHX. Cotreatment with CHX was efficient in sensitizing the cells to FasR-, TNF-R1-, and TRAIL-R-mediated cell killing, respectively, demonstrating that the receptors were present and functional, and that receptor stimulation was able to promote cell death (Fig. 1).

**Adenovirus-mediated Expression of Constitutively Active MKK1 Rescues HeLa Cells from Fas, TRAIL, and TNF-induced Apoptosis**—Whereas ERK1/2 inhibition was not sufficient to sensitize the cells to stimulation by TNFα, there is still the possibility that ERK1/2 could act as a secondary survival pathway in the TNF-R1 signaling. Because HeLa cells can be sensitized to Fas, TNF, and TRAIL-mediated apoptosis by treatment with CHX, we asked whether the survival mechanism activated by ERK1/2 would be able to override the CHX-induced sensitization. For this purpose, we used the RAd-CA-MKK1-HA adenovirus for expression of hemagglutinin (HA)-tagged constitutively active (CA) MKK1, the ERK1/2 activator, into HeLa cells. The amount of virus particles necessary for infection of 90–100% of the cells was determined by using an adenovirus construct containing the LacZ gene, followed by X-gal in situ staining. Surprisingly, we observed that expression of CA-MKK1-HA was not as efficient under the same conditions of infection because only 25–35% of the cells expressed CA-MKK1-HA (Table I). However, a higher penetrance of CA-MKK1-HA-positive cells could be obtained by longer incubation after infection (Table I). As the aim was to study direct signaling effects without involvement of ERK-mediated transcriptional activation, a short time period after expression of the initiating signaling protein was used. Despite the relatively low penetrance of CA-MKK1-HA expression at these early time points, the percentage of positive cells in the sample was sufficient to detect the effects of CA-MKK1-HA by microscopy and Western blot analysis (Figs. 2, and 5). Cells were sensitized to apoptosis by CHX, subjected to Fas, TNF, and TRAIL treatments, respectively and analyzed by fluorescence microscopy. This method enabled us to segregate the cells that actually express CA-MKK1-HA from the negative cells. Representative micrographs clearly show that the apoptotic cells did not express MKK1 (Fig. 2A), whereas the HA-positive cells were not apoptotic. Quantitative data obtained by manual counting of apoptotic cells, as determined by Hoechst staining, confirmed that only a minute fraction of apoptotic cells (<2%) was found to be HA-positive (Table I). As a comparison to this assay, we counted surviving cells remaining on equal areas of each coverslip (Fig. 2B). Because TNFα alone did not induce apoptosis in HeLa cells, we used TNF-treated CA-MKK1-HA expressing cells as a positive control to calculate the percentage of survival after the respective treatments (Fig. 2B). The results show that expression of CA-MKK1-HA rescued the cells from CHX-induced sensitization. Taken together, because the protection occurred shortly after infection when the expressed kinase did not have time to induce newly synthesized proteins and also in the presence of CHX, these data show that ERK1/2-mediated protection is independent of protein synthesis. Furthermore, the effect of CA-MKK1-HA expression not only counteracted the CHX sensitization, but it also protected against the normally occurring apoptosis induced by TRAIL alone (Fig. 2B). Interestingly, activation of ERK1/2 was also able to rescue the cells from TNF-induced apoptosis in CHX-sensitized cells. The survival data also provided a control showing that the adenovirus by itself did not affect our results because the RAdLacZ-transfected cells reacted in a similar way as the nontransfected population in Fig. 2B as well as untransfected cells (data not shown).

**The ERK1/2 Pathway Is Independent from the NF-κB Anti-apoptotic Pathway**—Triggering of the TNF-R1 is generally known to activate the NF-κB signaling cascade, a major anti-

### Table I

**The expression of HA-MKK1-CA in control cells and in DR-stimulated apoptotic cells**

For all samples presented in the table, the cells were first infected for 16 h with Ad-CA-MKK1-HA. After removal of the virus, the cells were incubated as follows. 1) The percentages of control cells positively stained for HA were counted 10 and 32 h after infection. 2) The percentages of HA-positive apoptotic cells were counted after 10 h in the presence of the respective DR agonists and CHX (see also Fig. 2).

| Counted apoptotic cells | HA-positive cells % |
|-------------------------|---------------------|
| Control (10 h)          | 783                 | 26.8            |
| Control (32 h)          | 365                 | 70.1            |

| Counted apoptotic cells | Apoptotic HA-positive cells % |
|-------------------------|------------------------------|
| Fas + CHX (10 h)        | 821                          | 1.0              |
| TNF + CHX (10 h)        | 822                          | 1.9              |
| TRAIL + CHX (10 h)      | 803                          | 1.8              |
Fig. 2. Expression of constitutively active MKK1 rescues HeLa cells from Fas, TNF, and TRAIL-induced apoptosis. An adenovirus construct was used to transfer the CA-MKK1-HA mutant gene in HeLa cells. Control cells and cells infected with RAd-CA-MKK1-HA were treated with anti-Fas, TNFα, or TRAIL (100 ng/ml each), with or without CHX (5 μM) or PD98059 (30 μM) for 10 h. A, micrographs of the samples show the CA-MKK1-HA expression (HA) and nuclear staining (Hoechst) of the same area. Examples of apoptotic cells, detected by their fragmented nuclear morphology, are shown with white arrows. These cells clearly do not express the constitutively active MKK1. E, HeLa cells plated on coverslips and infected with either RAd-CA-MKK1-HA or RAdLacZ as control were subjected to treatments with TNFα alone or TNFα, anti-Fas, and TRAIL with CHX. After fixation, the cells were stained for the HA tag of the CA-MKK1-HA mutant together with nuclear staining (Hoechst). Surviving cells from LacZ (black boxes), CA-MKK1-HA-negative (hatched boxes) and -positive (white boxes) cells were counted. The graph represents mean of survival compared with survival of the TNFα-treated samples.

Fig. 3. Only TNF strongly activates NF-κB. HeLa cells were treated for 15 min, 1 h, 3 h, and 6 h with anti-Fas, TNFα, or TRAIL-L (100 ng/ml each). The NF-κB activity was measured by electrophoretic mobility shift assay with a specific oligonucleotide probe. A representative autoradiograph is shown. As reported previously, TNF rapidly activates NF-κB, whereas neither Fas nor TRAIL has any effect on the transcription factor.

apoptotic pathway of the TNF-R1. TRAIL-R1, TRAIL-R2, and Fas-R have also been suggested to activate NF-κB under specific conditions and treatments (44–46). The activation steps involve induction of a kinase cascade comprising NIK and IKK, resulting in phosphorylation of the NF-κB inhibitor IκB and release of the transcription factor (47). Some reports suggest that NIK could in some situations be replaced by MEKK1 (48, 49). We wanted to know whether the difference in sensitivity toward stimulation of the respective receptors was because of activation of NF-κB. As expected from earlier studies (6, 20), TNF induced a marked binding activity of the NF-κB transcription factor with rapid kinetics (Fig. 3). However, neither Fas nor TRAIL activated NF-κB, thereby providing an explanation of why inhibition of ERK1/2 activation did not sensitize HeLa cells to TNF-induced apoptosis. Furthermore, the results suggest that the ERK1/2 survival mechanism would be independent from NF-κB activation.

All Death Receptors Can Activate ERK1/2—Because the resistance mechanism rendering a tumor cell line insensitive to DR-induced apoptosis could be caused by a high basal level of ERK1/2 in those cells, we studied the activation state of ERK1/2 in HeLa cells after receptor triggering. We treated the cells with anti-Fas, TNFα, or TRAIL for different time periods. The activation of ERK1/2 was determined by Western blot using a phospho-ERK1/2 antibody, which specifically recognizes the activated form of the kinase (Fig. 4A). Stimulation of Fas-R, TNF-R1, as well as TRAIL-R rapidly induced ERK1/2 phosphorylation. The activation of ERK1/2 appeared 5 min after treatment and was down-regulated after 1 h. The overall results show that the survival mechanism is not constitutive to the cell but rather activated by the apoptotic stimuli in itself. Because ERK1/2 activation could be mediated by caspses in the same way as indicated for the stress-activated protein kinases (SAPK; Refs. 37, 50), we tested the effect of the general caspase inhibitor Z-VAD-fmk on Fas-induced ERK1/2 phosphorylation. Although Z-VAD-fmk was an efficient inhibitor Fas-R-induced cleavage of caspase-8 (Fig. 4B), it clearly did not affect Fas-R-mediated activation of ERK (Fig. 4A).

Activation of ERK1/2 Blocks the Apoptotic Cascade Above the Level of Caspase-8—Cleavage and activation of caspase-8 is an early step in the apoptotic process triggered by the Fas-R, TNF-R1, and TRAIL-R, occurring at the level of DISC recruitment (10, 12, 15). The full-length procaspase is first cleaved once, releasing an intermediate 43-kDa fragment, which is further processed into the active 18-kDa fragment (51). To find out at what level activation of the ERK1/2 pathway would stop the cell death cascade, we infected HeLa cells with RAd-CA-MKK1-HA and observed whether procaspase-8 would still be cleaved into the active form. In nontransfected cells, the amount of cleaved caspase in the absence of sensitization is consistent with the amount of apoptotic cells measured earlier in the same conditions (Figs. 5 and 2A), suggesting that in resistant cells the apoptotic signal does not reach the activation of caspase-8 but is stopped at an earlier stage. Sensitization of the cells to DR-induced apoptosis by CHX induced a massive appearance of p18 and disappearance of the full-length procaspase-8. However, when CA-MKK1 is expressed, the amount of active fragment diminishes, and more procaspase-8 can be detected (Fig. 5).

DISCUSSION

The ERK1/2 pathway is of major importance in controlling cellular differentiation and growth (52–54), and it has also
MKK1-HA and control cells were treated for 10 h with anti-Fas, TNF or TRAIL-mediated caspase activation. Constitutively active ERK1/2 kinase inhibits Fas, TNF, and TRAIL-induced apoptosis. The phosphorylation state of ERK1/2 in HeLa cells was detected by Western blotting with a phosphospecific ERK1/2 antibody after 5 min, 15 min, 1 h, and 4 h of anti-Fas, TNFα or TRAIL treatment. Fas-induced activation of ERK1/2 was also followed for 5 min, 15 min, and 1 h in the presence of 20 μg/ml of the caspase inhibitor Z-VAD-fmk. The same samples were probed with ERK2 antibody as a loading control. B, control of the activity of Z-VAD-fmk by caspase-8 Western blot.

Fig. 4. Fas, TNF, and TRAIL activate ERK1/2. A, the phosphorylation state of ERK1/2 in HeLa cells was detected by Western blotting with a phosphospecific ERK1/2 antibody after 5 min, 15 min, 1, and 4 h of anti-Fas, TNFα or TRAIL treatment. Fas-induced activation of ERK1/2 was also followed for 5 min, 15 min, and 1 h in the presence of 20 μg/ml of the caspase inhibitor Z-VAD-fmk. The same samples were probed with ERK2 antibody as a loading control. B, control of the activity of Z-VAD-fmk by caspase-8 Western blot.

Fig. 5. Activation of ERK1/2 by adenovirus-based transfer of constitutively active ERK1/2 kinase inhibits Fas, TNF, and TRAIL-mediated caspase activation. HeLa cells expressing CA-MKK1-HA and control cells were treated for 10 h with anti-Fas, TNFα or TRAIL (100 ng/ml each) and sensitized to apoptosis by CHX (5 μM). Caspase activation was detected by Western blotting as appearance of the active p18 fragment of caspase-8, as well as disappearance of the proform.

been shown to act as an important modulator of various apoptosis-inducing signals in different systems (32, 55, 56). Our interest was to study whether this signaling cascade is involved in the signaling from other DRs, in addition to its established role as a regulator of FasR signaling. Although we and others described the ERK1/2 pathway as a mechanism preventing cell death induced by the FasR (34–36, 57, 58), the involvement of ERK1/2 in protection against TNF or TRAIL receptor-induced cell death has not been established so far, except for a study suggesting that ERK1/2 is involved in FGF-2-mediated protection against TNFα-induced apoptosis (35). We now provide evidence that ERK1/2 controls the responses from the other DRs too. ERK1/2 is likely to represent a mode of apoptosis regulation, which would be important especially during dynamic situations, when cells have to rapidly switch off the apoptotic signaling machinery. This inhibitor mechanism would then act in concert with inhibitor proteins, such as FLIP. The involvement of several regulatory pathways provides a multifaceted control system to direct the signals from these receptors.

Differences between Death Receptors in the Response to Receptor Activation and Inhibition of Mitogenic Signaling—HeLa cells have been shown to express the FasR, the TNF-R1, the TRAIL-R1, and the TRAIL-R2, each of them containing a death domain and able to induce apoptosis (45). However, there are some fundamental differences between the signaling responses elicited by activation of the respective receptors. As we show in this article, triggering of the receptors induce different responses in HeLa cells. Whereas HeLa cells were completely resistant to both Fas and TNF, they showed some sensitivity toward TRAIL-mediated apoptosis, although the degree of survival was still higher than in other tested TRAIL-sensitive cell lines (data not shown). In addition, ERK1/2 inhibition could sensitize HeLa cells to Fas and TRAIL killing, whereas it did not affect the resistance of the cells to TNF-mediated apoptosis. These results suggest differences in the modulation of DR responses. Interestingly, the responses we observed to correlate with the known physiological functions of each receptor. The primary function of the FasR has long been characterized as induction of apoptosis in different situations that include the immune response and regulation of the immune system. It has been suggested that tumor cells have developed several strategies to escape the immune surveillance by turning down this apoptotic pathway (59, 60), one strategy clearly being ERK-dependent protection. The TNF-R1, however, seems to be mainly involved in inflammatory responses, through activation of the NF-κB transcription factor (61), which diverts its signaling from the death pathway in most cell lines (6). Recent studies suggest that the TRAIL-Rs, the more novel of the four DRs, are especially important in removing virus-infected cells, as well as tumor cells (62–64), which would explain the higher sensitivity of HeLa cells toward this receptor. The existence of two DRs both activated by TRAIL renders the interpretation more delicate, as the resulting effect observed is most likely a combination of different responses from the respective TRAIL-Rs, rather than one single and redundant effect from both receptors or an effect from one receptor alone.

Several Survival Pathways Can Modulate Reinforce the Sensitivity of the Cells at a Given Time—TNF-R1 has been known more generally to direct its signaling toward transcription rather than apoptosis, as mentioned above, mainly by activating the transcription factor NF-κB, whereas both FasR and the TRAIL receptors are considered to be primarily apoptosis-directed, although they have also been suggested to have the capacity to activate NF-κB (17, 65, 66). It has been suggested that a Fas and TRAIL-mediated FADD-dependent activation of NF-κB in HeLa cells could be triggered by CHX, combined to caspase inhibition to prevent apoptosis, by way of an unknown CHX-sensitive factor. This experimental setup is, however, far from the physiological situation (45, 46). Our system reflects the more common responses of these receptors, as only TNFα was able to induce activation of the transcription factor NF-κB. This would explain why inhibition of ERK1/2 activation was not sufficient to induce TNF-mediated apoptosis, whereas it did
sensitize cells to Fas and TRAIL killing. However, TNF-induced apoptosis could be triggered by CHX cotreatment, and expression of CA-MKK1-IA was able to protect the cells in the same way as it could protect against Fas and TRAIL-mediated apoptosis. This is interesting, because it shows that ERK1/2 has a generally protective effect on DR-induced cell death, which could be used under specific conditions, when DR responses have to be rapidly modulated. It is interesting to speculate that, in case of failure of the main anti-apoptotic pathway, when NF-kB is not functional, the ERK1/2 pathway could take over the protection system. Additional protein synthesis-dependent anti-apoptotic factor(s) seem to be involved in DR signaling, considering the fact that CHX was able to sensitize the cells to Fas and TRAIL. FLIP has been suggested as a candidate for such a factor (46). The protective effect of ERK1/2, however, is independent of protein synthesis, as cells can be rescued by CA-MKK1 even in the presence of CHX.

Mechanism and Function of ERK1/2-mediated Survival—We have shown earlier that the ERK1/2 activation, which protects cells from apoptotic signal generated by the FasR, originated from activation of the receptor itself (38). We show here that the same applies to TNF-R1 and TRAIL-R, i.e. each receptor rapidly activates ERK1/2 upon stimulation. The fact that Fas-induced ERK1/2 activation was more rapid and transient in the current study than in the previous one, is likely to be explained by the difference in the Fas antibody used in the experiment.

The mechanism of DR-mediated ERK1/2 activation is not yet understood. A recent study suggests that FLIP could mediate rapid activation of ERK1/2 by recruitment of Raf-1 to the Fas DISC (67). Additional candidates for a role in ERK1/2 activation are the 14-3-3 proteins (68). Moreover, 14-3-3 has been implicated in Raf-1-mediated mitotic response (69). Concerning the downstream targets of ERK1/2 activation in this context, our data suggest that the apoptotic cascade is stopped either at an early stage before activation of the initiating factor caspase-8, or at the level of caspase-8. In a previous study (35), we showed that although the ERK1/2-mediated protection is active, the FasR-induced DISC can be assembled without all activation of caspase-8. Therefore, the apoptotic signal is inhibited either at the level of caspase-8 or at some upstream step of the feedback amplification loop, such as that involving cleavage of Bid and activation of cytochrome c release. The exact mechanism remains, however, to be investigated.

Intervention by ERK1/2 in the proapoptotic signaling cascades mediated by each of the three DRs adds to the similarities observed among the members of this family of proteins. It is interesting to note that the strong resemblance between the complexes assembled around the receptors do not prevent the differences in final functions. The complexity of the signals involved at the level and downstream of the respective DISCs allow precise modulation of the outcome and differences in the functions with possibility of redundancy. Although many interacting pathways related to DR stimulation have been deciphered, much remains to be resolved, both in normal cells as well as in cancer cells, in which disruption of some apoptotic signaling pathways have reduced the susceptibility to be killed by the immune system. Understanding the mechanism of ERK1/2-mediated protection both in normal and in pathological situations could lead to a better understanding of pathologic conditions related to defects in the apoptotic machinery.

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