Fas- or Ceramide-induced Apoptosis Is Mediated by a Rac1-regulated Activation of Jun N-terminal Kinase/p38 Kinases and GADD153*

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In the present study, we show that Fas receptor ligation or cellular treatment with synthetic C₆-ceramide results in activation or phosphorylation, respectively, of the small G-protein Rac1, Jun N-terminal kinase (JNK)/p38 kinases (p38-K), and the transcription factor GADD153. A signaling cascade from the Fas receptor via ceramide, Ras, Rac1, and JNK/p38-K to GADD153 is demonstrated employing transfection of transdominant inhibitory N17Ras, N17Rac1, or JNK/p38-K, or treatment with a specific p38-K inhibitor. The critical function of this signaling cascade is indicated by prevention of Fas- or C₆-ceramide-induced apoptosis after inhibition of Ras, Rac1, or JNK/p38-K.

Programmed cell death or apoptosis has been identified as a conserved and fundamental active cellular mechanism occurring under a range of physiological and pathological conditions (1–3). Programmed cell death is characterized by distinct morphological changes of the cell, including nucleus condensation and fragmentation, membrane blebbing, or formation of apoptotic bodies (4). A variety of stimuli have been identified to induce apoptosis in different cell types, for example stimulation of cells via the TNF, CD95/Fas/Apo1, or nerve growth factor receptor, treatment of cells with UV irradiation, reactive oxygen intermediates, heat shock, ceramides or cytotoxic drugs, infection by some viruses, or withdrawal of growth factors (5–17).

Apoptosis in mature lymphocytes can be induced by cellular stimulation via the Fas receptor (11, 13) belonging to the family of the nerve growth factor/TNF receptors (5), which are important in the regulation of apoptosis, proliferation or differentiation (5, 14). The major function of the Fas receptor/Fas ligand system seems to be the regulation of the peripheral immune response (18, 19). Thus, mutations of the Fas receptor or its ligand result in the defects of lpr and gld mice, respectively, characterized by lymphadenopathy, lympho-accumulation, and autoimmune organ failure (20, 21). Recently, mutations of the Fas receptor have been suggested as a mechanism for some human immunodeficiencies and the T-cell deficiency of human immunodeficiency virus might be due to a pathological stimulation via the Fas receptor (22–24). Several components of the death machinery have been identified; in particular, proteases of the ICE/Ced-3-family have been shown to be important in Fas triggered apoptosis (25, 26). Recently, it was demonstrated that the Fas receptor associates via its death domain, which exhibits significant homology with an intracellular domain of the TNF receptor and is required for induction of apoptosis (27), with FADD, TRADD, or RIP (11, 28, 29). FADD in turn binds to FLICE/MACH-1 (30, 31). FLICE/MACH-1 contains an ICE/Ced-3-like protease domain, which seems to be activated upon ligation and trimerization of the Fas receptor by a conformational change of FLICE/MACH-1 (30, 31). FLICE/MACH-1 then transmits the activation signal to ICE and CPP32, finally triggering cell death (30, 31). Recent studies suggest that caspases regulate the release of ceramide (32), a molecule that has been shown by us (9) and others (7) to be released upon Fas receptor stimulation implying that sphingomyelinases are downstream targets of caspases. Ceramides are known stimuli of apoptosis and a function of Jun N-terminal kinase (JNK) activation in the apoptotic effect of ceramides has been recently demonstrated (16).

Several other molecules have been shown to be involved in apoptosis, in particular the proto-oncogenes Myc, Abl, p120GAP, Fos, or Ras (9, 33–40). For example, knock-out mice of p120GAP show dramatic apoptosis of neurons in the developing brain (35), inhibition of Ras blocks Fas- or ceramide-induced programmed cell death in Jurkat T-lymphocytes (9), and expression of Myc triggers cell death in T-lymphocytes or fibroblasts (34, 37). Further molecules activated upon Fas receptor triggering are neutral and acidic sphingomyelinases (41), phospholipase A₂ (41), NFkB (42), Jun kinases (43–45), tyrosine and serine/threonine kinase (46, 47), the tyrosine phosphatase FAP (48), and Rac proteins (49).

In the present study, we investigated downstream effector molecules of Ras and Rac proteins upon Fas receptor stimulation. Activation of Jurkat cells via the Fas receptor or with synthetic ceramides resulted in a Ras- and Rac1-dependent stimulation of JNK and p38-K. The functional significance of the Fas- and ceramide-initiated signaling pathway from Ras via Rac to JNK/p38-K is indicated by an almost complete inhibition of Fas- or C₆-ceramide-mediated programmed cell death after transfection of the cells with transdominant inhibitory peptides.
Ras, Rac1, or an inhibitory Jun construct (Tam67) and simultaneous treatment with a specific pharmacological blocker (SB203580) of p38-K suggesting an important function of the described signaling pathway in the regulation of Fas-mediated programmed cell death.

MATERIALS AND METHODS

Cell Culture and Stimulation—All reagents were purchased from Sigma, Deisenhofen, Germany, if not otherwise cited. Human leukemic Jurkat cells were grown in RPMI 1640 medium supplemented with 10% fetal calf serum, 10 mM Hepes (pH 7.4), 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol (all from Life Technologies, Inc., Eggenstein, Germany.) For activation, cells were washed twice in Hepes-buffered saline (H/S: 132 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM CaCl2, 0.7 mM MgCl2, 0.8 mM MgSO4) and incubated at 37 °C with 2 μg/ml monoclonal anti-human Fas antibody (clone CH-11, Diavanna-Immunotech, Hamburg, Germany) or 5 μM C6-ceramide (Biomol, Hamburg, Germany) for the indicated times.

Inhibition of Ras, Rac, JNK, or p38-K—Co-transfection of Jurkat cells with transdominant inhibitory pEF-N17ras, pCEV-N17raco1, pRc/CMV-tam67 (each 50 μg/20 × 106 cells) or vector control (pEF, pCEV) with an expression vector for CD20 (pRc/CMV-cd20) (10 μg) was performed as described previously (9). N17Ras, N17Rac1 or Tam67 have been shown previously to inhibit the function of Ras, Rac, or JNK, respectively (17, 49, 50). Briefly, cells were electroporated using a BATX electroporator at 5 pulses (99 μF, 250 V). 12 h later, viable cells were purified by Ficoll gradient centrifugation and cultured for an additional 24 h. CD20 + cells were then selected by incubation with 50 μg/ml antibody (anti-CD20 monoclonal antibody, Dianova, Hamburg, Germany) (60 min, 4 °C), three washes, and further incubation (60 min, 4 °C) with magnetic beads coated with a sheep anti-mouse immunoglobulin (Dynal, Hamburg, Germany) (25). Since the ratio of 5:1 for pEF-N17ras, pCEV-N17raco1, or pRc/CMV-tam67 drives expression of N17Ras, N17Rac1, or Tam67 in any CD20 + cell, the selection of CD20 + cells permits effective sorting for N17Ras- or N17Rac1-expressing cells. The fraction of CD20 + positive and thus N17Ras- or N17Rac1-transfected cells was determined by flow cytometry of the cells with [H]thymidine as described previously (9). Aliquots of the labeled cells were counted prior and after sorting via magnetic beads. These experiments showed that approximately 10% of all cells were CD20 + positive. Non-specific binding of the cells to the magnetic beads did not exceed 0.5% of all cells, as determined by incubation of cells with an irrelevant monoclonal mouse anti-IgG antibody. Immunocomplexes were immobilized on agarose-coupled protein A/G; immunoprecipitates were stripped by a 45-min incubation in 20 mM Tris (pH 6.8), 2% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 0.5% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 125 mM NaCl, and 1% Triton X-100. GADD153 was immunoprecipitated from unlabeled, stimulated, or unstimulated cells for equal amounts of protein in all lanes. In addition, GADD153 was immunoprecipitated from the supernatant of all cells were CD20 + positive. Nonspecific binding of the cells to the magnetic beads did not exceed 0.5% of all cells, as determined by incubation of cells with an irrelevant monoclonal mouse anti-IgG antibody. Immunocomplexes were immobilized on agarose-coupled protein A/G; immunoprecipitates were stripped by a 45-min incubation in 20 mM Tris (pH 6.8), 2% SDS, 0.5% sodium deoxycholate, 125 mM NaCl, and 1% Triton X-100. GADD153 was immunoprecipitated from unlabeled, stimulated, or unstimulated cells for equal amounts of protein in all lanes. In addition, GADD153 was immunoprecipitated from unlabeled, stimulated, or unstimulated cells.
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7.5), 1% Triton, 12.5 mM β-glycerophosphate, 0.5 mM EGTA, 7.5 mM MgCl₂, 0.5 mM NaF, 0.5 mM Na₃VO₄, centrifuged, and recombinant GADD153 and 10 μCi/sample [γ-³²P]ATP were added to the supernatants. The samples were then incubated at 37 °C for 20 min, and the reaction was stopped by addition of 5 μl of boiling SDS sample buffer and 5% β-mercaptoethanol. The samples were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by autoradiography and laser densitometry.

Apoptosis—Fas-induced cell death was determined by metabolic labeling of Jurkat cells for 12 h with 10 μCi/sample [³²P]ATP were added to the supernatants. The samples were then incubated at 37 °C for 20 min, and the reaction was stopped by addition of 5 μl of boiling SDS sample buffer and 5% β-mercaptoethanol. The samples were separated by 10% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and analyzed by autoradiography and laser densitometry.

RESULTS

We recently demonstrated an activation of the Ras signaling pathway upon Fas receptor triggering or cellular treatment with ceramides (9). To identify downstream targets of this signaling pathway initiated by Fas or ceramide, we tested the activation of JNK/p38-K. Fas receptor triggering resulted in an approximately 15-fold stimulation of JNK/p38-K activity (Fig. 1, A and B). The stimulation of JNK or p38-K after Fas was comparable to the activation of the kinases upon cellular treatment with C₆-ceramide (Fig. 1, A and B). The activity of JNK or p38-K was determined by phosphorylation of the substrates GST-c-Jun or GST-ATF-2, respectively. The concentrations of 5 μM C₆-ceramide used in the present study has been shown to result in an intracellular concentration of 10–100 nmol/nmol of lipid (51). These concentrations are physiologically relevant, since they are also obtained upon Fas receptor ligation or serum deprivation (51). The activation of JNK/
FIG. 2. Fas (A) or C₆-ceramide (B) induce an activation of Rac1. Jurkat cells were metabolically labeled for 4 h and stimulated with anti-Fas (2 µg/ml) or C₆-ceramide (5 µM) for the indicated time. Rac1 was immunoprecipitated from the lysates, and bound guanine nucleotides were eluted from the immunoprecipitates, separated by TLC, and analyzed by autoradiography. The results show a long-lasting activation of Rac1 upon ligation of the Fas receptor, which is comparable with the stimulation of Rac1 after cellular treatment with C₆-ceramide. Activation of Rac1 was repeated three times. Immunoprecipitates using an irrelevant polyclonal control rabbit antiserum did not reveal any unspecific immunoprecipitation of a small G-protein (c = control immunoprecipitates).

p38-K by Fas or C₆-ceramide was similar to the stimulation of JNK or p38-K by previously reported stimuli, in particular heat shock, sorbitol, or PMA (Fig. 1C) (16, 52–55). The inactive stereoisomer dihydro-C₂-ceramide did not induce stimulation of JNK or p38-K, showing the specificity of the activation.

The increase of JNK or p38-K activity correlated with a phosphorylation of the two kinases upon Fas receptor ligation or cellular treatment with C₆-ceramide (Fig. 1D). Phosphorylation was determined by incubation of JNK or p38-K immunoprecipitates with a phospho-JNK or phospho-p38-K-specific antibody and development using the ECL technique. Reprobing the blots with anti-JNK or anti-p38-K antibodies revealed similar amounts of proteins in all lanes (shown in the smaller blots below).

To elucidate the mechanism of JNK and p38-K activation by Fas and C₆-ceramide, we tested whether Ras and Rac proteins, which have been shown to be upstream regulators of JNK/p38-K (52, 56), regulate the stimulation of JNK/p38-K after anti-Fas or C₆-ceramide treatment. Rac proteins have been implied as intermediates transmitting signals from Ras to Jun N-terminal kinase or MAP-kinase kinase, which then activate JNK or p38-K, respectively (52, 56). A stimulation of Ras by Fas receptor ligation or cellular treatment with ceramides has been previously shown by us (9). Further studies from our group showed a Ras-dependent activation of Rac1 and Rac2 upon Fas receptor triggering (49). Here, we provide a detailed analysis of the kinetics of Rac activation upon Fas receptor ligation and show that Rac is rapidly stimulated by Fas receptor triggering peaking 10 min after stimulation (Fig. 2A). The activity of Rac1 after Fas remained increased for more than 40 min after anti-Fas treatment.

The activation of Rac1 upon Fas receptor ligation is mimicked by cellular treatment with C₆-ceramide (Fig. 2B); Rac1 activation peaked approximately 10 min after addition of C₆-ceramide and remained stimulated for more than 40 min after addition of C₆-ceramide (Fig. 2B). The specificity of C₆-ceramide-mediated Rac1 stimulation is indicated by the finding that the biologically inactive stereoisomer dihydro-C₂-ceramide does not trigger Rac1 activation (data not shown).

To analyze the involvement of Ras and Rac proteins in JNK/p38-K activation by Fas or C₆-ceramide, Jurkat cells were transiently co-transfected with an expression vector for transdominant inhibitory N17Ras or N17Rac1 and CD20. The mutants have a very high affinity to GDP (50) and bind endogenous guanine nucleotide exchange factors, preventing the activation of endogenous Ras or Rac1. N17Rac1 inhibits the activation of both Rac1 and Rac2 after Fas receptor stimulation as demonstrated previously (49). Expression of the B-lymphocyte antigen CD20 in transfected cell permits efficient purification of N17Ras- or N17Rac1-expressing cells (9). Inhibition of endogenous Ras by N17Ras or Rac by N17Rac1 almost completely prevented activation of JNK or p38-K after Fas receptor triggering (Fig. 3, A and B) or cellular treatment with ceramides (Fig. 3, C and D). The crucial role of Ras and Rac proteins for Fas- or C₆-ceramide-initiated JNK and p38-K activation is also indicated by an inhibition of the phosphorylation of JNK and p38-K upon Fas receptor or C₆-ceramide triggering by transfection of N17Ras or N17Rac1 (data not shown). The efficiency of N17Ras or N17Rac1 expression and the purification process has been shown previously (49) and is demonstrated in Fig. 3E. These data indicate an approximately 90% inhibition of Ras or Rac1 activation after anti-Fas triggering (Fig. 3E and Ref. 49). In summary, these data suggest that Ras and Rac proteins are upstream regulators of JNK/p38-K upon Fas receptor stimulation or treatment with C₆-ceramides.

To determine the significance of the observed signaling cascade for Fas- or ceramide-induced programmed cell death, we measured the effect of an inhibition of endogenous JNK/p38-K, Rac1, or Ras by transfection of transdominant inhibitory constructs or treatment with pharmacological inhibitors on apoptosis after anti-Fas or C₆-ceramide (Fig. 4). Cells were co-transfected with either Tam67, N17Rac1, or N17Ras and CD20 or treated with the specific p38-K inhibitor SB 203580, metabolically labeled with [³H]thymidine, purified by sorting via CD20, and stimulated with anti-Fas or C₆-ceramide, and apoptosis was determined as described above. In all experiments apoptosis was also detected by typical morphological changes after trypan blue staining. The experiments show that inhibition of JNK and p38-K, endogenous Rac1, or Ras prevents Fas- and C₆-ceramide-induced apoptosis by more than 80%, indicating the significance of the signaling cascade from Ras via Rac proteins to JNK/p38-K for Fas- and ceramide-triggered programmed cell death. Single inhibition of JNK or p38-K did not change Fas- or ceramide-induced apoptosis. Control experiments with SB 203580 showed that the inhibitor reduced p38-K activity after Fas receptor stimulation by approximately 90% (data not shown). The efficiency of Tam67 transfection was tested by measuring the up-regulation of c-Jun and IL-2 production upon treatment of the cells with PMA (10 ng/ml) and ionomycin (500 ng/ml). Both activation markers have been previously shown to be inhibited by expression of Tam67 (45). Induction of c-Jun or release of IL-2 were almost completely inhibited in cells transfected with Tam67 and sorted via CD20, whereas control transfected cells responded normally with c-Jun expression and IL-2 synthesis (data not shown). These results indicate a sufficient transfection and expression of Tam67 in CD20-sorted Jurkat cells.

To gain insight into the function of JNK/p38-K activation upon Fas receptor stimulation, we measured the phosphorylation of the transcription factor GADD153. GADD153 is a known target of JNK and has been implied in the regulation of gene expression and/or cell cycle regulation (57–59). Fas receptor ligation (Fig. 5A) or ceramide treatment (Fig. 5B) induced a
strong phosphorylation of GADD153, which was almost completely inhibited by transfection of transdominant inhibitory N17Rac1 or of the transdominant inhibitory c-Jun construct Tam67 and simultaneous pretreatment with the p38-K inhibitor SB 203580 (Fig. 5, C and D). Similar results were obtained using an anti-phosphoserine antibody showing an approximately 10-fold stimulation of GADD153 phosphorylation (data not shown).

The in vivo phosphorylation of GADD153 could be mimicked by incubation of recombinant GADD153 with cell lysates obtained from cells stimulated via the Fas receptor or with C₆-ceramides (Fig. 5E).

The data show that the phosphorylation of GADD153 is regulated by a signaling cascade via Rac proteins and JNK/p38-K.

**DISCUSSION**

The results of the present and previous studies (9, 49) show a signaling cascade from the Fas receptor via the small G-proteins Ras and Rac to JNK/p38-K and the transcription factor GADD153. Activation of this cascade seems to be necessary for Fas- or C₆-ceramide-mediated cell death, since genetic or pharmacological inhibition of Ras, Rac, or JNK/p38-K prevents the induction of apoptosis. Furthermore, the results identify GADD153 as a new, Ras-, Rac-, and JNK/p38-K-regulated downstream effector of Fas and synthetic ceramide. This notion is supported by the finding that incubation of recombinant GADD153 with cell lysates in vitro from Fas or C₆-ceramide stimulated cells results in a phosphorylation of GADD153.

In the present study, inhibition of Ras and Rac proteins was achieved by transient transfection of transdominant inhibitory
Inhibition of Rac proteins, JNK, and p38-K prevents Fas- or C6-ceramide-induced programmed cell death. Cells were labeled for 12 h with \( ^{3} \text{H} \)thymidine prior to induction of apoptosis. Rac proteins or JNK were inhibited by transient co-transfection of transdominant inhibitory N17Rac1 or Tam67 and CD20. CD20\(^{+} \) cells were sorted and treated with anti-Fas (2 \( \mu \text{g/ml} \)) or C6-ceramide (5 \( \mu \text{M} \)) for 4 h. p38-K was inhibited by preincubation with the specific p38-K inhibitor SB 203580 (10 \( \mu \text{M} \)). DNA fragmentation was determined by binding of intact DNA to glass fiber filters, followed by liquid scintillation counting (9). Apoptosis was also measured by staining an aliquot of the cells with trypan blue and determining typical morphological changes. The two methods measuring apoptosis or DNA fragmentation showed very similar results.

constructs. These mutants, N17Ras or N17Rac1, have a very high affinity to GDP and therefore bind the endogenous GDP/GTP-exchange factors, preventing the activation of endogenous Ras or Rac1/2 (49, 50). To identify transfected cells, we performed a co-transfection with an expression vector for CD20, which is a B-cell antigen not expressed in T-lymphocytes. This co-transfection technique permits efficient sorting of transfected cells as described previously (9). The blockade of JNK/p38-K stimulation by genetic inhibition of Ras and Rac indicates that Fas receptor-induced JNK and p38-K activation is mediated by a sequential activation of Ras and Rac proteins. Our data show that Rac1 is longer (more than 40 min) active than JNK/p38-K. Since Rac1 is able to interact with different downstream effector molecules linking Rac1 to cytoskeleton regulation (60, 61), JNK/p38 activation (52, 56), or reactive oxygen release (62, 63), it is likely that JNK/p38-K are not the only target molecules of Rac1 upon Fas receptor ligation. Thus, it might be possible that Rac determines in a temporally organized way the activity of different downstream molecules, and therefore the activation time of these molecules might be shorter than of Rac1.

Inhibition of JNK was achieved by transfection of a transdominant inhibitory c-Jun construct, TAM67, which has been used previously to inhibit cell death triggered by the nerve growth factor-p75 receptor (17). The Tam67 c-Jun mutant lacks the N-terminal transactivation domain, which includes the JNK binding site, and functions as a transdominant interfering mutant. p38-MAP kinase was inhibited by incubation of the cells with the pharmacological inhibitor SB 203580, which is considered to be a specific inhibitor of p38-K (64). Tam67 transfection prevented the up-regulation of c-Jun or release of IL-2 after T-cell stimulation. Likewise, SB 203580 almost completely inhibited the activity of p38-K upon Fas receptor triggering. This demonstrates the efficiency of the Tam67 transfection and the kinase inhibitor treatment. Since, in accordance with other studies (45, 65), single inhibition of JNK or p38-K did not prevent Fas- or ceramide-triggered programmed cell death, whereas combined inhibition of JNK and p38-K significantly reduced Fas- or ceramide-mediated apoptosis, the activation of both kinases seems to be required for induction of apoptosis in Jurkat cells. Likewise, ceramide-initiated JNK activation has been demonstrated to be essential for the induction of apoptosis after ionizing radiation, UV light, heat shock, ceramide, TNF\(\alpha \), or \( \text{H}_{2}\text{O}_{2} \) (16). However, in this study, Tam67-transfected U937 cells were almost completely resistant to induction of apoptosis by the mentioned stimuli. Therefore, it might be possible that the expression levels of JNK versus p38-K determine whether both kinases are required for induction of apoptosis or only one of the two kinases. Thus, in some cell types Tam67 might be sufficient to prevent apoptosis by ceramide, Fas, or stress, whereas in other cells, e.g. Jurkat, inhibition of both JNK and p38-K is required to prevent apoptosis. JNK/p38-K do not seem to be important in all forms of apoptosis, since transfection with Tam67, treatment with SB 203580, or combination of the two inhibitors did not prevent apoptosis in Jurkat cells incubated with thapsigargin (1 \( \mu \text{M} \)), a microsomal Ca\(^{2+} \) ATPase inhibitor.\(^2\)

Our notion of an activation of JNK and p38-K by Fas receptor ligation is supported by previous studies showing that Fas stimulates these kinases (16, 43–45, 65, 66). The time course of JNK activation after Fas seems to depend on the dose of the antibody used for stimulation, since treatment with 1–2 \( \mu \text{g/ml} \) anti-Fas results in JNK activation after already 10 min (Ref. 43 and present study), whereas lower concentrations (30–100 ng/ml) require longer incubation times (60–90 min) to induce JNK stimulation.

In summary, the studies suggest an important function of JNK/p38-K for Fas- or ceramide-mediated programmed cell death.

The activation of a signaling cascade from the Fas receptor via Ras or Rac proteins and JNK/p38-K to GADD153 may have several functions important for Fas- or ceramide-triggered pro-

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\(^2\) B. Brenner, U. Koppenhoefer, C. Weinstock, O. Linderkamp, F. Lang, and E. Gulbins, unpublished data.
GADD153 may result in a change of GADD153 activity in programmed cell death; JNK/p38-K-regulated phosphorylation of GADD153 may promote its ability to induce cell cycle arrest. In this case, the cell cycle might be arrested during a Fas-sensitive phase permitting Fas receptor ligation to trigger apoptosis. Finally, it might be possible that ceramide and Fas induce growth arrest and/or DNA damage, which may result in an altered activity of GADD153. Thus, the phosphorylation of GADD153 would be secondary to the induction of apoptosis. This possibility seems to be unlikely since the phosphorylation of GADD153 may result in an altered activity of GADD153. Thus, the phosphorylation of GADD153 upon Fas receptor ligation is already observed 10 min after Fas receptor ligation or C6-ceramide treatment. This time seems to be rather short to mediate a significant DNA damage resulting in the regulation of GADD153. However, since the physiological function and regulation of GADD153 is only poorly characterized, an alternative function is certainly possible and elucidation of the exact role of GADD153 in Fas-induced apoptosis has to be performed in future studies.

It has been shown that Fas-induced cell death does not require new mRNA or protein synthesis (70). Our experiments do not exclude the possibility that cells already in a certain state of the cell cycle are able to undergo apoptosis upon Fas receptor ligation, whereas in a cell population with heterogeneous cell cycle status the cell cycle progression regulated via Ras, Rac, JNK/p38-K, and GADD153 might be necessary to achieve complete induction of apoptosis by Fas.

Alternatively, JNK and p38-K may have cytosolic targets involved in the apoptosis of lymphocytes upon Fas receptor ligation. In particular, p38-K has been implied in the phospho-

Figure 5. A and B, GADD153 is phosphorylated after stimulation via the Fas receptor ligation (A) or treatment with C6-ceramide (B). Jurkat cells were metabolically labeled with [32P]P, stimulated with anti-Fas (2 μg/ml) (A) or C6-ceramide (5 μm) (B) for the indicated time, GADD153 was immunoprecipitated, samples were separated by 10% SDS-PAGE and blotted, and phosphorylation was analyzed by autoradiography. Reprobing of the blots revealed similar amounts of GADD153 in all lanes. C and D, inhibition of Rac or JNK and p38-K prevents phosphorylation of GADD153 upon Fas receptor (C) triggering or C6-ceramide (D) application. Jurkat cells were cotransfected with N17Rac1 or Tam67 and CD20 and sorted for CD20+ cells. Tam67-transfected cells were also incubated with the p38-K inhibitor SB 203580. Cells were stimulated with anti-Fas (2 μg/ml) (C) or C6-ceramide (5 μm) (D), and the phosphorylation of GADD153 was determined as above. E, cell lysates from Fas- or ceramide-activated cells phosphorylate GADD153 in vitro. Jurkat cells were stimulated via the Fas receptor (2 μg/ml) or with C6-ceramide (5 μm) and lysed, and the lysates were incubated with recombinant GADD153 in the presence of [γ-32P]ATP. Lysates from stimulated cells contain a kinase activity phosphorylating GADD153. Control immunoprecipitates (c) using irrelevant rabbit antibodies show the specificity of GADD153 immunoprecipitation. The experiments were repeated three times.
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