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Fas-induced Apoptosis Is Mediated by Activation of a Ras and Rac Protein-regulated Signaling Pathway*

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Fas induces apoptosis in lymphocytes via a poorly defined intracellular signaling mechanism. We and others have previously demonstrated the involvement and significance of a signaling cascade from the Fas receptor via sphingomyelinases and ceramide to Ras in apoptosis (Gulbins, E., Bissonette, R., Mahboubi, A., Nishioka, W., Brunner, T., Baier G., Baier-Bitterlich, G., Byrd, C., Lang, F., Kolesnick, R., Altman, A., and Green, D. (1995) *Immunity* 2, 341; Cifone, M. G., DeMaria, R., Roncali, P., Rippo, M. R., Azuma, M., Lanier, L. L., Santoni, A., and Testi, R. (1994) *J. Exp. Med.* 180, 1547–1552; Gill, B. M., Nishikata, H., Chan, G., Delovitch, T. L., and Ochi, A. (1994) *Immunol. Rev.* 142, 113–126). Here, we demonstrate an activation of the small G-proteins Rac 1 and Rac 2 after Fas receptor triggering. Expression of a transdominant inhibitory Ras mutant (N17Ras) prevents Rac 1 and Rac 2 stimulation, suggesting a signaling cascade from the Fas receptor via Ras to Rac 1 and Rac 2. Genetic and pharmacological inhibition of Ras or Rac 1 and Rac 2 stimulation blocks Fas-induced apoptosis, pointing to an important function of a Ras and Rac protein-regulated signaling pathway in Fas-mediated programmed cell death.

Programmed cell death or apoptosis is a highly conserved active cellular mechanism characterized by cell shrinkage, chromatin condensation, and nuclear fragmentation (4). Apoptosis occurs in many physiological or pathophysiological conditions and has been shown to be fundamental for the normal development of multicellular organs (5–7). Several stimuli, including the tumor necrosis factor (8, 9), Fas/ApoI/CD95 receptor (10, 11), reactive oxygen intermediates (ROI)[1] (12), ionizing radiation (13), ceramides (1, 2, 14), or daunorubicin (15) have been demonstrated to induce apoptosis in different cell types. Apoptosis in lymphocytes can be induced by cellular stimulation via the Fas receptor (10, 16–20) belonging to the family of the nerve growth factor/tumor necrosis factor receptors (9), which are important in the regulation of apoptosis, proliferation, or differentiation (8, 9). The Fas receptor seems to be particularly important in the homeostasis of mature, peripheral lymphocytes (17, 18), whereas the function of Fas in the thymus remains unknown. Mutations in the Fas receptor or its ligand result in lymphadenopathy, lymphoaccumulation, and autoimmune organ failure of lpr or gld mice and expression of a Fas transgene in T cells corrects the lpr defect (19–21). Recently, mutations of the Fas receptor have been also implied 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).

We and others demonstrated previously that activation via the Fas receptor stimulates the acidic sphingomyelinase leading to synthesis of ceramide (1–3) and activation of the small G-protein p21Ras (1). The activation of Ras is essential for Fas-induced apoptosis, since inhibition of Ras blocks apoptosis; however, the downstream targets of Ras upon Fas triggering are unknown. Further results indicate an activation of neutral sphingomyelinases, phospholipase A_2, and interleukin converting enzyme-like proteases upon Fas receptor triggering (25–27).

Here, we demonstrate a Ras-dependent activation of the small G-proteins Rac 1 and Rac 2 upon Fas receptor triggering. Genetic inhibition of Ras, Rac 1, and Rac 2 by expression of transdominant inhibitory N17Ras, N17Rac1, Rac 1, and Rac 2 antisense oligonucleotides, or cellular treatment with *Botulinus* C3 exoenzyme prevent Rac 1 and Rac 2 stimulation as well as programmed cell death after Fas receptor stimulation showing the significance of a Ras and Rac protein-regulated signaling cascade for apoptosis.

MATERIALS AND METHODS

Cells, Stimulation, and Lysis—Human leukemic Jurkat cells were grown in RPMI 1640 supplemented with 10% fetal calf serum, 10 mM Hepes, pH 7.4, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 μM nonessential amino acids, 100 units/ml penicillin, 100 μg/ml streptomycin, and 50 μM β-mercaptoethanol. For activation, cells (2×10^6 or 20×10^6 per sample for total cell lysates or immunoprecipitations, respectively) were washed twice in Hepes-buffered saline (H/S, 132 mM NaCl, 20 mM Hepes, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, and 0.8 mM $MgSO_4$) and incubated at 37 °C with 2 μg/ml monoclonal anti-human-Fas-antibody (clone CH-11; Dianova-Immunotech, Germany) for the indicated times. Stimulation was terminated by lysis of the cells (see below).

Transfection Assay, Phosphorothioate Antisense Oligonucleotides, and Botulinus C3 Exoenzyme Application—Cotransfection of Jurkat cells with transdominant inhibitory pEF/N17ras, pCEV/N17rac 1 (each 50 μg/20×10^6 cells) or vector control (pEF and pCEV) with an expression vector for CD20 (pRc/CMV-cd20) (10 μg) was performed as described previously (1). Briefly, cells were electroporated using a BTX-electroporation apparatus at five pulses (99 μs) and 500 V. Twelve 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 anti-CD20 mAb (Dianova) (60 min at 4 °C), washed three times, and further incubated (60 min at 4 °C) with mag-

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[1] The abbreviations used are: ROI, reactive oxygen intermediate; JNK, c-*jun*-NH_2-kinase.

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netic beads coated with a sheep anti-mouse Immunoglobulin (Dynal) (1). Since the ratio of 5:1 pEF/N17ras or pCEV/N17rac 1:pRc/CMV-cd20 drives expression of N17Ras or N17Rac 1 in any $CD20^+$ cell, the selection of $CD20^+$ cells permits effective sorting for N17Ras- or N17Rac 1-expressing cells. Rac 1-, Rac 2-, and Ras activity or apoptosis were determined in $CD20^+$ enriched cells.

To inhibit protein expression of Rac 1 and Rac 2, Jurkat cells (1×10^6) were incubated for 18 h with phosphorothioate antisense oligonucleotides (5′-ACTTGATGGCCTGCA-3′; 10 μM or 60 μM) or control oligonucleotides (5′-TGGCTATGCCACATG-3′; 10 μM or 60 μM, a random mix of the same nucleotides within the antisense molecule) (28) or left untreated. To inhibit Rac proteins independently of antisense oligonucleotides, cells were treated with *Botulinus* C3 exoenzyme, which ADP-ribosylates Rac proteins (29), a modification which has been shown to inhibit the function of Rac proteins. To this end, 100 μg of *Botulinus* C3 exoenzyme (Calbiochem, San Diego, CA) were electroporated into 5×10^6 Jurkat cells at 420 V and 125 microfarads as described previously (1, 30). Prior to electroporation, Jurkat cells were labeled with [^{3}H]thymidine to allow determination of apoptosis (see below). The electroporation method has been previously shown by us and others to be a very efficient method to introduce proteins into cells (1, 30). After electroporation, cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum and allowed to recover for 1 h at 37 °C as described previously (1).

Ras and Rac Assay—Jurkat, N17Ras, N17Rac 1, pCEV, or pEF and CD20 cotransfected cells were washed twice in phosphate-free Dulbecco's modified Eagle's medium, resuspended in the same medium complemented with 10% dialyzed fetal calf serum, and labeled for 4 h with 1 mCi/ml $^{32}P_i$. Cells were stimulated with anti-Fas (2 μg/ml) for the indicated time or left untreated. Stimulation was terminated by lysis in 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 20 mM $MgCl_2$, 450 mM NaCl, and 100 μg each of aprotinin and leupeptin (lysis buffer). Lysis with SDS and sodium deoxycholate permits efficient extraction of Rac proteins, even after translocation to the Triton X-100-insoluble fraction (see below). Nuclei and cell debris were removed by centrifugation ($15{,}000 \times g$) at 4 °C for 15 min. Ras, Rac 1, or Rac 2 were immunoprecipitated from the lysates at 4 °C for 45 min using the monoclonal anti-Ras-antibody Y13–259 or a polyclonal, affinity-purified anti-Rac 1 or anti-Rac 2 antibody, respectively. The anti-Rac 1 or anti-Rac 2 antibody is against a carboxyl-terminal domain and do not cross-react with Rac 2 or Rac 1, respectively. The immunoprecipitates were collected by further incubation with goat anti-rat or anti-rabbit immunoglobulin-conjugated agarose beads. Precipitates were washed seven times in lysis buffer, and bound nucleotides were eluted in 1 mM EDTA at 68 °C for 20 min. The samples were then centrifuged at $15{,}000 \times g$ for 5 min, and the nucleotides in the supernatant were separated on PEI-cellulose plates (Machery & Nagel, Dueren, Germany) with 0.75 M KH_2PO_4, pH 3.5. The thin-layer chromatography plates were analyzed by autoradiography, the spots corresponding to GDP and GTP were scrapped from the plate, and radioactivity was determined by liquid scintillation counting. Activation of Ras, Rac 1, or Rac 2 is expressed by an increase of the GTP:(GDP+GTP) ratio.

Translocation of Rac Proteins—Activation of Rac 1 and Rac 2 correlates with a translocation of the proteins from the Triton X-100-soluble cell fraction to the Triton X-100-insoluble cell fraction (31). To determine a translocation of Rac proteins upon Fas receptor triggering, cells (1×10^6/sample) were stimulated with 2 μg/ml anti-Fas, lysed in 1% Triton X-100, 125 mM NaCl, 25 mM Tris, pH 8.0, 10 mM EDTA, and 10 mM NaF. After centrifugation, the supernatant was used as a Triton X-100-soluble cell fraction, and the pellet was used as a Triton X-100-insoluble cell fraction. DNA was sheared by passing the sample through a 27-gauge needle. Samples were separated on a 15% SDS-polyacrylamide gel electrophoresis and blotted, and Rac 1, Rac 2, Ras, or Rho A (Santa Cruz Biotechnology, Santa Cruz, CA) was detected by incubation with the corresponding antibody, followed by horseradish peroxidase-protein G and the ECL system.

Apoptosis—The effect of transdominant inhibitory N17Ras or N17Rac 1 transfection and a blockade of Rac 1 and Rac 2 protein expression by antisense oligonucleotides or C3 exoenzyme treatment on Fas-induced cell death was determined on cells metabolically labeled for 12 h with 10 μCi/ml [^{3}H]thymidine (8.3 Ci/mmol; DuPont NEN) (1). Cells were washed, aliquoted, and incubated with anti-Fas (200 ng/ml) or left untreated. Cell death was determined after 3 h by DNA fragmentation and trypan blue staining. Briefly, cells were disrupted by one cycle of freezing at −20 °C, and thawing, unfragmented genomic DNA was collected by filtration through glass fiber filters (Pharmacia Biotech Inc.) and counted by liquid scintillation (1). Results are expressed as %DNA fragmentation ± S.D. compared to control samples. Experiments were done in triplicate and repeated three times. Determination of cell death by trypan blue staining showed the same results.

RESULTS

Several studies suggest an activation of the Ras signaling pathway in Fas- or tumor necrosis factor-induced apoptosis (1, 15). Since Rac proteins might be downstream targets of Ras (32, 33) and thus involved in Fas-triggered apoptosis, we tested the activation of Rac 1 and Rac 2 by anti-Fas antibody treatment. The specificity of the monoclonal CH-11 anti-Fas antibody in the Jurkat system has been shown previously (1). To measure Ras activity, Rac 1 or Rac 2 were immunoprecipitated from $^{32}P_i$ metabolically labeled Jurkat cells, and Rac 1- or Rac 2-bound guanine nucleotides were determined (Fig. 1*A*). The results show a rapid activation of Rac 1 and Rac 2 after anti-Fas treatment, indicated by an approximately 5-fold increase in the GTP:(GTP+GDP) ratio of Rac 1- and Rac 2-bound nucleotides.

To examine the relationship between Ras and Rac proteins in T cells, Jurkat cells were transiently cotransfected in a ratio of 5:1 with transdominant inhibitory N17Ras using an expression vector with an elongation factor promoter (pEF-N17ras) and an expression vector for CD20 (pRc/CMV-cd20) (1). Since the B-lymphocyte antigen CD20 is not expressed in T lymphocytes, this cotransfection technique permits selection for N17ras-transfected cells via the surface marker CD20. CD20-positive cells were purified 36 h after transfection by incubation with an anti-CD20 monoclonal antibody (50 μg/ml) and magnetic beads. The enriched N17ras-positive cells were stimulated with 2 μg/ml anti-Fas. Activity of Rac 1 and Rac 2 was determined as above in Rac 1 or Rac 2 immunoprecipitates from $^{32}P_i$ metabolically labeled cells (Fig. 1*B*). Expression of N17Ras inhibited stimulation of Rac 1 and Rac 2 by Fas, whereas expression of the vector alone did not influence Rac 1 or Rac 2 activation after Fas receptor triggering (Fig. 1*B*). As demonstrated previously, N17Ras expression almost completely inhibited anti-CD3-mediated Ras stimulation, demonstrating the efficiency of N17Ras expression (data not shown). Anti-CD3 triggering has been described as a potent stimulus of Ras (34). The transfection of N17Ras has also been shown to inhibit mitogen-activated protein kinase stimulation by Fas and activation of an interleukin 2 promoter chloramphenicol acetyltransferase gene construct by TCR/CD3 receptor triggering (1); both activation events have been demonstrated to be regulated by Ras. Expression of transdominant inhibitory N17Ras did not inhibit Fas-induced overall tyrosine phosphorylation (1), indicating a specific inhibition of Ras and not a general block of cell signaling by N17Ras.

Upon stimulation, Rac 1 and Rac 2 translocate from the Triton X-100-soluble cell fraction to the Triton X-100-insoluble cell fraction (31). Thus, to confirm activation of the Rac proteins after anti-Fas, the Triton X-100 solubility of Rac 1 and Rac 2 was examined. Western blots of the Triton X-100-soluble and Triton X-100-insoluble fractions of anti-Fas-stimulated or unstimulated Jurkat cells revealed a rapid and significant translocation of Rac 1 and Rac 2 from the Triton X-100-soluble to the Triton X-100-insoluble cell fraction after stimulation (Fig. 1*C*), supporting the notion of an activation of Rac proteins by Fas receptor triggering. The translocation of the two Rac proteins was almost completely prevented in cells transfected with N17Ras, whereas the cells transfected with vector control showed a strong translocation of Rac 1 or Rac 2 from the Triton X-100-soluble fraction to the Triton X-100-insoluble fraction. The purity of the Triton X-100-insoluble, "membranous" fraction is indicated by the absence of the tyrosine kinases Fyn and Zap, two Triton X-100-soluble proteins, in this fraction prior or

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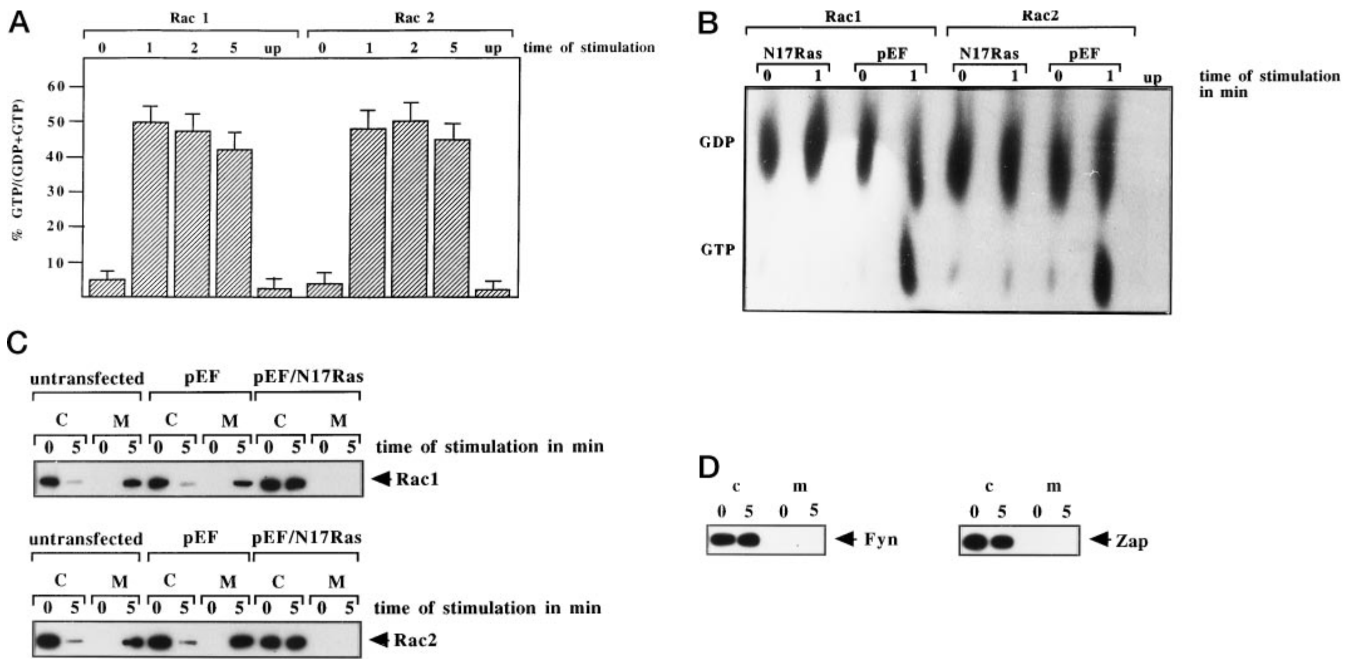

FIG. 1. In *A,* Fas receptor triggering rapidly stimulates the small G-proteins Rac 1 and Rac 2. In *B,* the activation of Rac 1 and Rac 2 by Fas is mediated by a Ras-regulated signaling pathway. Inhibition of endogenous Ras using transdominant inhibitory N17Ras prevents Fas-induced Rac 1 and Rac 2 stimulation, whereas the control vector pEF does not affect Fas-induced cell activation. In *C,* upon stimulation via Fas, Rac 1 and Rac 2 translocate from the Triton X-100-soluble cell fraction to the Triton X-100-insoluble cell fraction. Translocation of the two proteins is inhibited in cells transfected with transdominant inhibitory N17Ras. Rac 1 and Rac 2 were immunoprecipitated from untreated, N17Ras, or pEF-transfected Jurkat cells metabolically labeled for 4 h with 1 mCi/ml $^{32}P_i$ (1). Cells were stimulated with anti-Fas (2 μg/ml) for 1 min or the indicated time and lysed in 25 mM Tris-HCl, pH 7.5, 1% Triton X-100, 0.2% SDS, 0.5% sodium deoxycholate, 20 mM $MgCl_2$, 450 mM NaCl, and 100 μg each of aprotinin and leupeptin. This buffer permits efficient extraction of Triton X-100-insoluble and Triton X-100-soluble Rac 1 and Rac 2. Nucleotides were eluted from washed precipitates, separated on a PEI-cellulose plate with 0.75 M KH_2PO_4, pH 3.5, and analyzed by autoradiography. *A* and *B* show the autoradiographies and the liquid scintillation analysis of the GDP and GTP spots. In *C,* translocation of Rac 1 and Rac 2 was determined by lysis of Fas-stimulated or unstimulated Jurkat-, pEF-, or N17Ras-transfected cells in 1% Triton X-100, 125 mM NaCl, 25 mM Tris, pH 8.0, 10 mM EDTA, and 10 mM NaF. The Triton X-100-soluble (*c*) or -insoluble (*m*) fractions were separated on SDS-polyacrylamide gel electrophoresis and transferred to Immobilon membranes; Rac 1 and Rac 2 translocation was detected by incubation with an affinity-purified anti-Rac 1 and -Rac 2 antibody (Santa Cruz), followed by ECL analysis. In *D,* the absence of the tyrosine kinases Fyn and Zap in the "membranous", Triton X-100-insoluble fraction prior or after Fas receptor triggering indicates the purity of this fraction. Shown is a Western blot of Fyn and Zap from the Triton X-100-soluble cell fraction and the Triton X-100-insoluble cell fraction.

after cellular stimulation via Fas. These data support the notion of a Ras-regulated activation of Rac 1 and Rac 2 upon Fas receptor triggering.

To study the significance of Ras and Rac protein activation for Fas-induced apoptosis, Ras and Rac proteins were inhibited by different genetic and pharmacological methods. Ras was blocked by transfection of Jurkat cells with transdominant inhibitory N17Ras (Fig. 2*A*). Rac 1 and Rac 2 were inhibited by transfection with transdominant inhibitory N17Rac 1, cellular treatment with Rac 1 and Rac 2 antisense oligonucleotides (10 μM), which suppressed expression of both Rac proteins, or by treatment with C3 exoenzyme, which inhibits Rac proteins by ADP-ribosylation (29) (Fig. 2*B*). Fas-induced cell death was almost completely prevented by either inhibition of Ras by N17Ras (Fig. 2*A*) or by blockade of Rac proteins by N17Rac 1 expression and treatment with antisense oligonucleotides or C3 exoenzyme (Fig. 2*B*), respectively. Analysis of Rac 1 and Rac 2 activity in cells transfected with transdominant inhibitory N17Rac 1 or vector control (pCEV) confirmed an almost complete inhibition of both Rac proteins by the transdominant inhibitory N17Rac 1 construct (Fig. 2*C*). Western blot analysis of total cell lysates showed a more than 80% reduction of Rac 1 and Rac 2 protein expression (Fig. 2*D*) by 18 h incubation with phosphorothioate antisense molecules (10 μM). Blotting with anti-Rho A or anti-Ras antibodies revealed that the antisense oligonucleotides seem to be specific for Rac mRNA. None of the controls had any effect on Fas-induced apoptosis. The experiments were repeated with a higher concentration (60 μM) of the Rac antisense oligonucleotides, revealing a similar inhibition of Fas-triggered programmed cell death (data not shown).

In conclusion, these results indicate a critical role of a Rac 1- and Rac 2-regulated signaling pathway in Fas-induced cell death.

DISCUSSION

These and previous experiments (1, 2) show a signaling cascade from the Fas receptor via sphingomyelinases, ceramide, and Ras to the small G-proteins Rac 1 and Rac 2. Genetic or pharmacological inhibition of Ras or Rac proteins prevents programmed cell death, demonstrating the significance of this pathway for Fas-induced apoptosis.

Rac 1 and Rac 2 may mediate apoptosis by activation of one or more of the following pathways:

(*a*) Rac proteins, which share approximately 92% homology, may regulate a signaling cascade via JNKK to JNK and p38-K (35, 36). JNK has been recently shown to be important for nerve growth factor receptor-, ceramide-, and Fas-induced apoptosis (37–39), and transfection of transdominant inhibitory JNK constructs prevented nerve growth factor receptor- or ceramide-triggered apoptosis. Since the Fas receptor triggers synthesis of ceramides via activation of an acidic sphingomyelinase (1–3), resulting in activation of Ras (1), Rac proteins may mediate the stimulation of JNK/p38-K by the Fas receptor. However, the molecular mechanism of JNK-induced cell death is unknown.

(*b*) Rac 1 and Rac 2 may induce changes of actin filaments.

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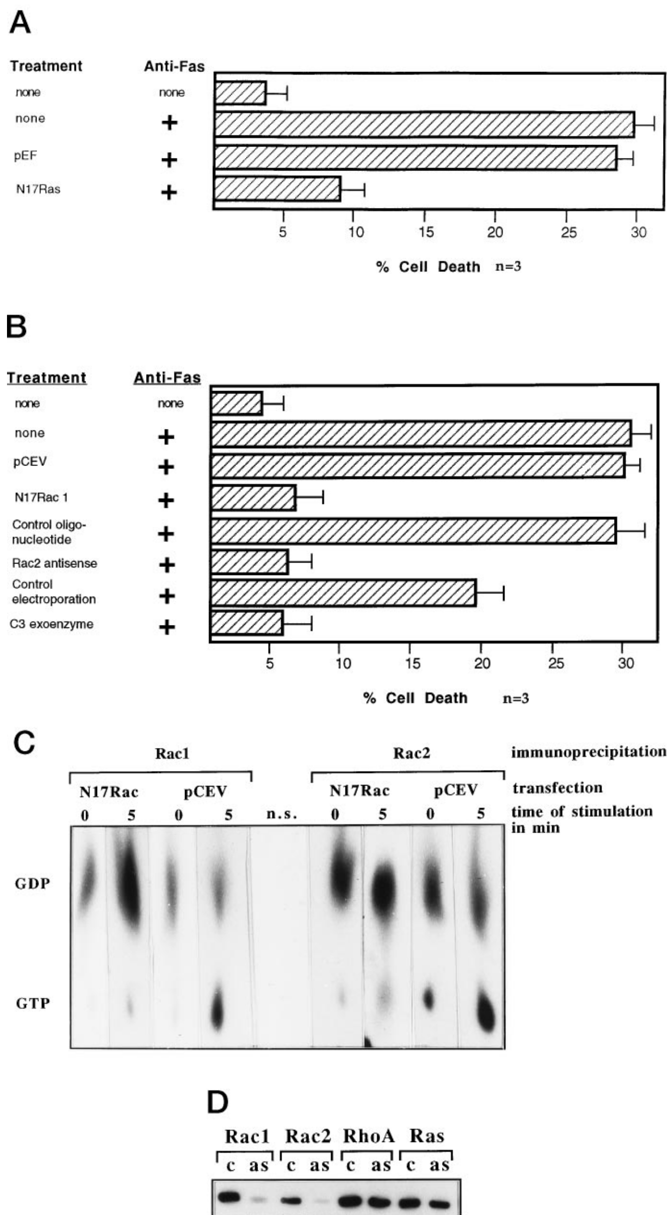

FIG. 2. **Inhibition of endogenous Ras by transfection of transdominant inhibitory N17Ras (*A*) or of Rac 1 and Rac 2 by transfection of transdominant inhibitory N17Rac 1, antisense oligonucleotides, or C3 exoenzyme treatment (*B*), respectively, prevent Fas-induced programmed cell death.** *A,* Jurkat cells were transfected with pEF-N17ras or pEF and pRc/CMV-cd20 as above. $CD20^+$-enriched cells were used to determine Fas-mediated apoptosis. *B,* to block Rac 1 and Rac 2, Jurkat cells (20×10^6) were cotransfected with N17Rac 1 and CD20 as described above. Next, the cells (1×10^6) were incubated for 18 h with phosphorothioate antisense oligonucleotides (5′-ACTTGATGGCCTGCA-3′; 10 or 60 μM) or control oligonucleotides (5′-TGGCTATGCCACATG-3′; 10 or 60 μM), or left untreated. To inhibit Rac 1 and Rac 2 by ADP-ribosylation, 100 μg of C3 exoenzyme (Calbiochem, San Diego, CA) were electroporated into 5×10^6 Jurkat cells at 420 V and 125 microfarads. After electroporation, cells were resuspended in RPMI 1640 supplemented with 10% fetal calf serum and allowed to recover for 1 h at 37 °C. In all experiments, [^{3}H]thymidine-labeled cells were collected, and DNA fragmentation was determined by filtration through glass fiber filters and liquid scintillation counting. All samples were also analyzed for the morphological changes of apoptosis by trypan blue staining. In *C,* the efficiency of the inhibition of endogenous Rac 1 and Rac 2 by transfection of transdominant inhibitory N17Rac 1 is shown by an almost complete blockade of Rac 1 and Rac 2 activation in N17Rac 1-transfected cells, whereas vector control-transfected cells show a 4–5-fold stimulation of Rac 1 or Rac 2, respectively, after Fas receptor triggering. In *D* is shown a Western blot of whole-cell lysates stained with anti-Rac 1 and anti-Rac 2 from phosphorothioate antisense (*as*)- or control oligonucleotide (*c*)-treated cells. Rac 1 or Rac 2 expression is reduced by approximately 80%. The expression of other small G-proteins (Rho A and Ras) is not changed by incubation with the Rac antisense oligonucleotides. Protein expression was determined by blotting whole-cell lysates with the corresponding antibody, followed by horseradish peroxidase-protein G and ECL.

Activation of Rac 1 has been shown to trigger polymerization of actin filaments in fibroblasts (32). Thus, Fas receptor triggering may induce a polymerization of actin filaments via Rac 1 and/or Rac 2. However, preliminary experiments of our group do not show an actin polymerization after Fas receptor triggering; instead, we observed a fragmentation of actin filaments after anti-Fas treatment. Thus, a regulation of the actin filaments by Fas via Rac proteins seems to be unlikely.

(*c*) Rac 1 and Rac 2 have been implied in the synthesis of O_2^- radicals (ROIs) in neutrophils (28, 40) and B lymphocytes (28). Since ROIs have been implied in apoptosis (41–43), a synthesis of ROIs by Fas via Rac 2 might be involved in Rac 2-regulated apoptosis. On the other hand, apoptosis has been shown to proceed under very low oxygen conditions (44) and may be independent of O_2^- radicals. Results from our group show a very transient synthesis of ROI upon Fas receptor triggering, which seems to be regulated by Rac 2. Since the observed synthesis of ROIs is very transient and low, the released ROIs probably do not directly damage the cell. They may stimulate further signaling molecules, *e.g.* kinases or proteases (45), involved in apoptosis.

It might be possible that the combined activation of Rac 1 and Rac 2 proteins by the Fas receptor enables the cell to activate several pathways, *e.g.* JNK/p38-K activation via Rac 1 and ROI synthesis via Rac 2.

In our experiments, Fas-mediated activation of Rac 1 and Rac 2 was determined by two different methods: (*a*) we measured directly the increase of GTP-binding to Rac 1 and Rac 2 after Fas receptor triggering; and (*b*) the translocation of Rac 1 and Rac 2 to the Triton X-100-insoluble cell fraction, which has been shown to correlate with an activation of Rac protein (31), was determined. Both methods indicate an activation of Rac 1 and Rac 2 strongly suggesting a stimulation of Rac proteins by Fas receptor triggering. Whether Fas-induced Rac 1 and Rac 2 activation is mediated by an activation of guanine nucleotide exchange factors or an inactivation of GAP proteins remains to be determined.

The regulation of Rac 1 and Rac 2 via Ras was demonstrated in cells transfected with transdominant inhibitory N17Ras. N17Ras has a very high affinity to GDP (46); thus, it binds guanine nucleotide exchange factors, *e.g.* Sos, and prevents activation of endogenous Ras (46). The cotransfection of N17Ras with CD20 allows sorting of N17Ras-transfected cells and analysis of Rac 1 or Rac 2 activity in an almost pure N17Ras-expressing population. The efficiency of the transfection and sorting process is shown by the finding that under typical conditions, less than 7% of the cells bind unspecifically to the CD20 antibody (1). This cotransfection technique allows us to postulate a signaling cascade from Ras to Rac proteins initiated by Fas receptor triggering.

Inhibition of Rac 1 and Rac 2 was achieved by three independent methods, *i.e.* blockade of Rac 1 and Rac 2 activation by transfection of a transdominant inhibitory N17Rac1 construct, inhibition of Rac 1 and Rac 2 protein expression by antisense oligonucleotides, and ADP-ribosylation of Rac proteins using *Botulinus* C3 exoenzyme. Transdominant inhibitory N17Rac 1 seems to inhibit Rac proteins by a similar mechanism as described above for N17Ras. Since Rac 1 and Rac 2 are very similar, it is likely that they are activated by the same or very similar GEFs, which are inhibited by N17Rac 1. The antisense oligonucleotides used in the present study have been previously

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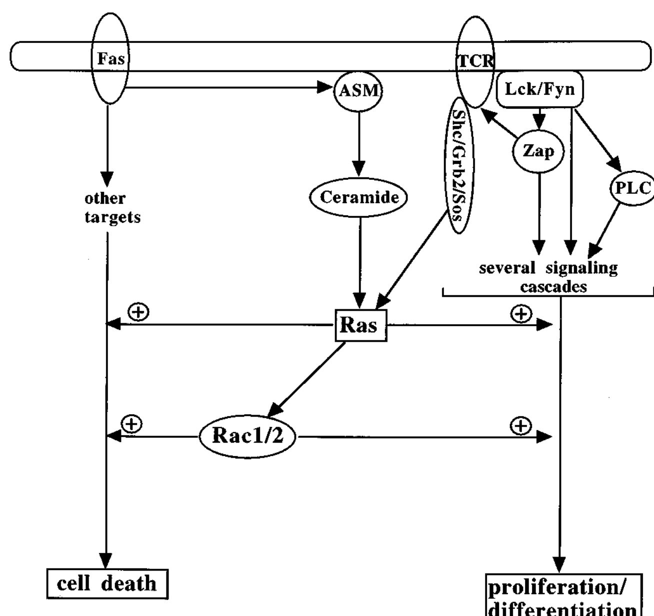

FIG. 3. **Model of function of Ras and Rac proteins in Fas-induced programmed cell death.** Several receptors are able to activate Ras and Rac proteins, which are regulating pathways specific for cell death or proliferation. Since these pathways may need a positive signal from Ras and Rac proteins to proceed, Ras and Rac proteins are able to control both cell death or proliferation.

shown to efficiently block Rac protein expression in B lymphocytes (28). The C3 exoenzyme was introduced in the cells by electroporation, a method which has been shown by us (1) and others (30) to allow efficient loading of cells with proteins. Control experiments with fluorescein isothiocyanate-labeled bovine serum albumin or fluorescein isothiocyanate-labeled anti-tubulin displayed loading of 70–80% of Jurkat cells after electroporation. Control electroporation or electroporation of bovine serum albumin had no impact on Fas-induced apoptosis. However, since ADP-ribosylation of other G-proteins by C3 exoenzyme may occur, the data with C3 exoenzyme should be interpreted in the context with the results of the experiments using N17Rac 1 transfection and antisense oligonucleotides. Since the three methods, which are completely independent ways to inhibit Rac proteins, prevent Fas-induced apoptosis, we suggest a crucial role of Rac proteins in Fas receptor-triggered programmed cell death.

Our results pointing to the importance of Ras and Rac proteins in Fas-induced apoptosis appear paradoxical in the light of the critical function of Ras and Rac proteins in cell proliferation (47–49). However, several molecules with functions in cell proliferation, including c-Myc (50, 51), c-Fos (52), Cdc-2 (53), p21Ras (54, 55), R-Ras (56), or p120GAP (57), are involved in the control of programmed cell death. A role of Ras in apoptosis is also indicated by the finding that activated Ras is able to trigger apoptosis in fibroblasts (54, 55). Therefore, Ras, Rac 1, and Rac 2 may function as factors that are able to influence or regulate both cell survival/differentiation and cell death pathways. The actual function of Ras and Rac 1 and Rac 2 may depend on co-signals provided from growth factor receptors, oncogenes, or receptors inducing programmed cell death. Thus, a certain set of signaling pathways induces apoptosis, whereas another set triggers proliferation. Some of these signaling pathways, *e.g.* the Ras pathway, are common, but the specific biological outcome, death or proliferation, is determined by the specific combination of these signaling pathways.

Alternatively, Ras and/or Rac proteins may positively regulate pathways leading to cell proliferation or apoptosis and allow these pathways to proceed (Fig. 3). Thus, Ras and/or Rac proteins may control apoptosis (or proliferation) and may be necessary for but not sufficient to induce apoptosis.

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