Requirement of an ICE-Like Protease for Induction of Apoptosis and Ceramide Generation by REAPER

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Genetic studies indicated that the *Drosophila melanogaster* protein REAPER (RPR) controls apoptosis during embryo development. Induction of RPR expression in *Drosophila* Schneider cells rapidly stimulated apoptosis. RPR-mediated apoptosis was blocked by *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk), which suggests that an interleukin-1β converting enzyme (ICE)–like protease is required for RPR function. RPR-induced apoptosis was associated with increased ceramide production that was also blocked by Z-VAD-fmk, which suggests that ceramide generation requires an ICE-like protease as well. Thus, the intracellular RPR protein uses cell death signaling pathways similar to those used by the vertebrate transmembrane receptors Fas (CD95) and tumor necrosis factor receptor type 1.

In genetic screens for abnormal cell death patterns in *Drosophila* embryos homozygous for specific chromosomal deletions, two genes, *reaper* (*rpr*) and *head involution defective*, have been identified that are involved in the control of apoptosis (programmed cell death) (*1*, *2*). In situ analysis and ectopic expression of *rpr* have demonstrated that the 65–amino acid RPR protein plays an important role in the induction of apoptosis in the developing *Drosophila* embryo (*2*). The mechanism by which RPR induces apoptosis is unknown. Genetic analysis of *Caenorhabditis elegans* has implicated the cysteine protease CED-3 in programmed cell death, and mammalian homologs of CED-3 have recently been implicated in apoptosis induced by Fas ligand and tumor necrosis factor α (TNF-α) (*3*). This growing family of mammalian Asp-directed cysteine proteases includes ICE, ICH1/NEDD2, ICH2/TX, CPP32/YAMA/apopain, and MCH2 (*4*–*9*). The involvement of these proteases in mammalian cell death has been established by the use of inhibitors such as the cowpox virus CrmA protein and certain peptide methyl ketones and peptide aldehydes that irreversibly bind to the active-site cysteine of the protease (*5*, *10*–*12*). Another enzyme that has been implicated in cell death by biochemical studies of mammalian apoptosis is sphingomyelinase, which hydrolyzes sphingomyelin to form phosphocholine and ceramide (*13*). Ceramide functions as a second messenger in the induction of apoptosis by TNF-α, Fas ligand, and ionizing radiation (*14*). RPR has some homology to the death domains of Fas and TNF receptor type 1 (TNFR-1) (*15*), which raises the possibility that these proteins share common signaling mechanisms. To study whether RPR, a small intracellular protein, uses cellular components similar to those used by the transmembrane receptors for Fas ligand and TNF-α, we established cell lines in which we could induce RPR expression and RPR-mediated cell death.

We stably transfected Schneider cells (S2) with a construct containing RPR complementary DNA (cDNA) under the control of a *Drosophila* metallothionein promoter (*16*). Treatment of metallothionein-RPR (Mt-RPR) cell lines with 0.5 mM $CuSO_4$ resulted in rapid induction of *rpr* mRNA and RPR protein (*17*) (Fig. 1). Small amounts of *rpr* message were detected as early as 20 min after the addition of $CuSO_4$. RPR protein was detected 60 min after the addition of $CuSO_4$; however, we could detect small amounts of RPR protein as early as 30 min after $CuSO_4$ addition by analyzing RPR immunoprecipitates. RPR expression was rapidly followed by the appearance of apoptotic cells, as judged by extensive membrane blebbing, shrinkage of cell body and nucleus, and Hoechst 33258 staining (*18*). After 90 min of $CuSO_4$ treatment, 50 to 60% of the cells showed extensive membrane blebbing, and this increased to >90% after 18 hours of treatment with $CuSO_4$. Vector control cells and parental cells did not respond to $CuSO_4$. Immunofluoresence staining showed that RPR was mainly cytosolic and appeared to be excluded from the nucleus (Fig. 2A) (*19*). The asynchronicity in the expression of RPR may be due to a difference in response to $CuSO_4$ during different stages of the cell cycle and could in part explain the somewhat heterogeneous death response.

We next tested whether RPR induces apoptosis through activation of proteases. In mammalian cells granzymes (*20*), ICE-like proteases (*3*), cathepsins (*21*), and calpain (*22*) all have been implicated in apoptosis. We assessed the effect of different protease inhibitor peptides on RPR-induced apoptosis by first incubating Mt-RPR cells with 50 μM concentrations of the following peptides: *N*-benzyloxycarbonyl-Val-Ala-Asp-fluoromethylketone (Z-VAD-fmk) (an ICE-like protease inhibitor), Z-Phe-Ala-fmk (Z-FA-fmk, a cathepsin B inhibitor), Z-Ala-Ala-Asp-chloromethylketone (Z-AAD-cmk, a granzyme B inhibitor), or *N*-acetyl-Leu-Leu-norleucinal (ALLN, a calpain-cathepsin inhibitor) (*16*, *23*, *24*). The sequences of these peptides were based on substrate cleavage sites; for instance, VAD is the ICE recognition and cleavage site in progenitor interleukin-1β, and peptides based on this sequence inhibit partially purified ICE- and Fas-mediated apoptosis (*12*, *24*). Cells were incubated with each peptide for 90 min and treated with 0.5 mM $CuSO_4$ for 2 hours; we then scored the number of apoptotic cells by counting the percentage of cells showing membrane blebbing. The ICE-like protease inhibitor Z-VAD-fmk completely blocked RPR-induced apoptosis, as confirmed by Hoechst 33258 staining (Fig. 2B). Z-VAD-fmk had no

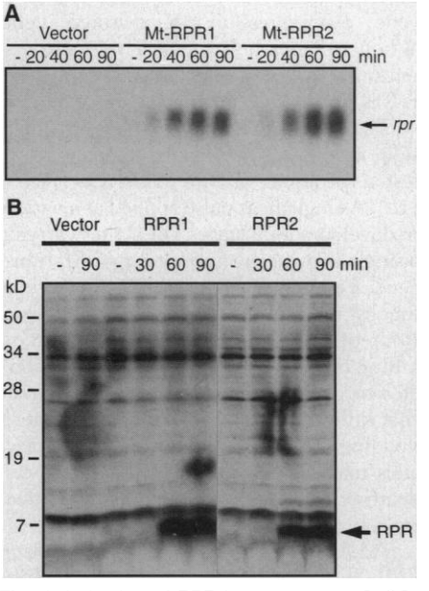

Fig. 1. Induction of RPR in response to $CuSO_4$ treatment. (**A**) Northern blot analysis of the induction of *rpr* mRNA in response to 0.5 mM $CuSO_4$ treatment of vector control, or two S2 cell lines stably transfected with the metallothionein-RPR constructs Mt-RPR1 and Mt-RPR2. (**B**) Protein immunoblot analysis of RPR protein expression after $CuSO_4$ treatment. Cells were treated as in (A). The bands in the upper portion of the gel are also stained with the preimmune serum, whereas RPR is not recognized by this control serum (*18*).

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effect on cell morphology, growth rate, or the induction of RPR protein expression. Other inhibitors tested either did not block the effect of RPR or, as in the case of Z-FA-fmk, augmented the effect of RPR expression on cell death. Thus, an ICE-like protease is required for RPR function.

Both TNF-α– and Fas ligand–induced apoptosis are associated with increased amounts of ceramide (*14*). We therefore tested whether RPR-induced cell death was accompanied by an increase in ceramide. Mt-RPR1 cells and control cells were treated with $CuSO_4$ for the indicated times; lipids were subsequently extracted and ceramide amounts were determined (*25*). There was a substantial increase in ceramide in response to $CuSO_4$ treatment in the Mt-RPR1 cells but not in control cells (Fig. 3A). Similar results were obtained with Mt-RPR2 and the Mt-RPR transfection pool (*18*). The increase in ceramide occurred shortly after the induction of RPR expression, as detected by immunoblotting of RPR immunoprecipitates (see insert, Fig. 3A). Increased ceramide was detected at the same time that changes in cell morphology were observed, which suggests that ceramide may be involved in transducing part of the death signal. We next examined the effect of Z-VAD-fmk and Z-FA-fmk on ceramide in RPR-expressing cells. Z-VAD-fmk completely blocked the increase in ceramide. In contrast, Z-FA-fmk augmented the RPR-induced increase in ceramide (Fig. 3B). These results indicate that the generation of ceramide requires one or more functional ICE-like proteases and is therefore a relatively late event in RPR-induced apoptosis. Our data do not indicate whether ceramide is a signaling molecule in RPR-induced apoptosis or is formed as a result of apoptosis.

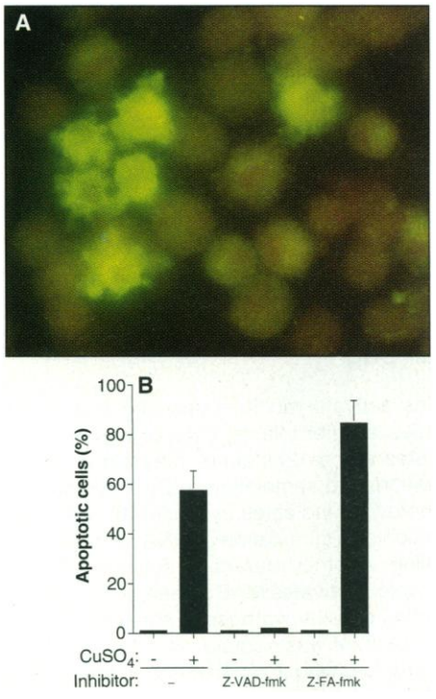

Fig. 2. Induction of RPR expression results in apoptosis. (**A**) Detection of RPR by immunofluoresence in Mt-RPR1 cells. Mt-RPR1 cells were treated for 90 min with $CuSO_4$. After fixation, the cells were stained with affinity-purified RPR peptide antibodies and antibody to rabbit IgG-FITC. The strongly positive cells show plasma membrane blebbing, which is typical for apoptotic cells. Vector control cells treated with $CuSO_4$ did not stain with the RPR antiserum, nor was the same staining pattern observed when preimmune serum was used (*18*). (**B**) RPR-induced apoptosis is blocked by inhibition of an ICE-like protease. Mt-RPR1 cells were not treated or were treated for 90 min with 50 μM Z-VAD-fmk (an ICE-like protease inhibitor) or 50 μM Z-FA-fmk (a cathepsin B inhibitor). Subsequently, $CuSO_4$ was added to a final concentration of 0.5 mM. The percent of apoptotic cells was determined after 120 min of $CuSO_4$ treatment by examination of at least 200 cells (n = 3).

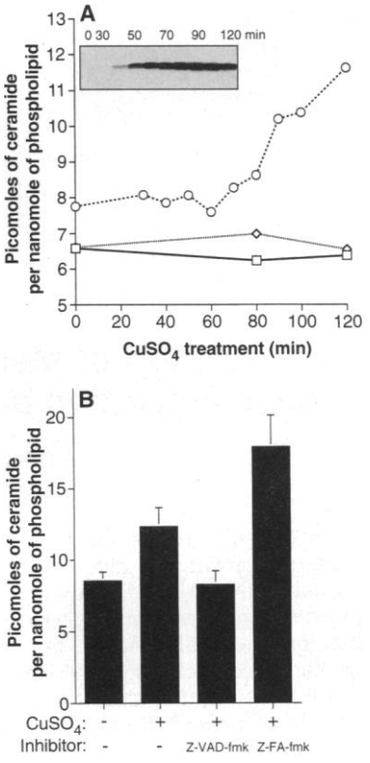

Fig. 3. RPR-induced apoptosis is associated with increased ceramide amounts. (**A**) Ceramide amounts increase shortly after the appearance of RPR. S2 cells (squares) and vector control cells (diamonds) were treated with 0.5 mM $CuSO_4$ for 0, 80, and 120 min, after which lipids were extracted as described (*25*). Mt-RPR1 cells (circles) were treated with $CuSO_4$ for the indicated times, after which the cells were harvested. Lipid extracts were obtained from four-fifths of the cells. The lipid extracts were used to determine ceramide amounts relative to the amount of total cellular phospholipid (*25*). The remainder of the samples were lysed so that the amount of RPR expression could be determined by immunoblotting of RPR immunoprecipitates. The insert shows the RPR amounts in Mt-RPR1 cells treated with $CuSO_4$ for the indicated times. (**B**) RPR-induced increases in ceramide are blocked in the presence of Z-VAD-fmk. Mt-RPR cells were not treated or were treated with 0.5 mM $CuSO_4$ in the absence or presence of 50 μM Z-VAD-fmk or 50 μM Z-FA-fmk. Cells were harvested and ceramide amounts were determined as described above (n = 2).

In mammalian cells, exogenous ceramide induces apoptosis that is indistinguishable from that induced by TNF-α. To test whether ceramide could mimic RPR-induced cell death, we treated S2 cells with a mixture of 50 μM C2-ceramide and 50 μM C6-ceramide (*25*). Treatment with these ceramides resulted in the death of 50% of the cells after 16 hours, as detected by trypan blue staining. Ceramide-induced cell death was not inhibited by the Z-VAD-fmk peptide, which indicates that exogenous ceramide does not act through the Z-VAD-fmk–sensitive ICE-like protease (*18*). However, cell deaths induced by RPR and ceramide were not equivalent. Ceramide-induced cell death did not involve shrinkage of the cells and plasma membrane blebbing, which suggests that ceramide may account for only part of the death signal. Alternatively, the ceramides we used are different from the endogenous ceramide induced by RPR and may have different biological effects. Finally, we cannot rule out the possibility that ceramide production is a consequence rather than a cause of apoptosis. In analogy with TNFR-1 and Fas, RPR-induced generation of ceramide is most likely the result of activation of a sphingomyelinase. However, RPR-induced de novo synthesis of ceramide, as has been reported during daunorubicin-induced apoptosis, is also possible (*26*).

We have shown here that RPR-induced apoptosis and increases in ceramide both require a functional ICE-like protease. The cytotoxic signals of both TNFR-1 and Fas can be blocked by ICE-like protease inhibitors as well (*11*, *12*). Our data suggest that the production of ceramide upon activation of these receptors may also depend on ICE-like proteases. Our findings also imply that even though RPR is a small intracellular molecule, it plays a proximal role in activating the biochemical processes culminating in apoptosis, and our findings indicate a high degree of conservation of these pathways among species and among various initiators of apoptosis.

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16. Schneider S2 cells were maintained in Schneider's *Drosophila* medium (Gibco) supplemented with 10% fetal bovine serum (FBS) (Hyclone) and penicillin-streptomycin at 27°C. RPR cDNA was cloned into an expression vector containing the *Drosophila* metallothionein promoter [T. A. Bunch, Y. Grinblat, L. S. B. Goldstein, *Nucleic Acids Res.* **16**, 1043 (1988)] and cotransfected with a plasmid containing the hygromycin resistance gene under the control of the *Drosophila* actin 5C promoter. Stable transfections of S2 cells were performed by the calcium phosphate method [M. Ashburner, *Drosophila; A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), pp. 142–145]. Because of the low adherence of the S2 cells, the transfection pool was grown up, and monoclonal cell lines were subsequently generated by limiting dilution (technique to subclone cells) in 96-well plates. The polyclonal transfection pool (Mt-RPR pool) and eight subclones (Mt-RPR1 to -8) were analyzed, and all gave similar results with regard to RPR induction and cell death in response to $CuSO_4$. In the rest of the study, we used the empty vector control pool, the Mt-RPR pool, and Mt-RPR1 and -2. For metallothionein promoter activation, cells were plated in Schneiders medium with 10% FBS, and 24 or 48 hours later, 0.5 M $CuSO_4$ was added to a final concentration of 0.5 mM. For the peptide inhibition experiments, cells were treated with 50 μM of the following peptides: Z-VAD-fmk, Z-FA-fmk, Z-AAD-cmk (Enzyme System Products, Dublin, CA), or ALLN (Sigma). After 90 min, $CuSO_4$ was added to a final concentration of 0.5 mM, and after 2 hours the percentage of apoptotic cells was determined by examination of at least 200 cells for the presence of plasma membrane blebs.
17. For the Northern (RNA) blot analysis, cells were treated with 0.5 mM $CuSO_4$ for the indicated times, after which total cellular RNA was isolated with the Ultraspec RNA isolation kit (Biotecx Laboratories, Houston, TX). Twenty-five micrograms of total RNA was used for the Northern blot analysis,which was performed according standard procedures. For the analysis of RPR protein, cells were treated as described above, and after a brief wash with ice-cold phosphate-buffered saline (PBS) the cells were lysed in Laemmli sample buffer. Fifty micrograms of protein was analyzed for the presence of RPR by immunoblotting with RPR peptide antiserum. The RPR peptide antiserum was raised in rabbits with the use of the COOH-terminal 15 amino acids of RPR (NH_2-CHPKTGRKSGKYRKP-COOH) (*27*), cross-linked to keyhole limpet haemocyanin (Animal Pharm Services, Healdsburg, CA). The RPR antiserum was affinity-purified on an RPR peptide column [E. Harlow and D. Lane, *Antibodies, A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1988), pp. 313–318].
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19. Vector control cells and Mt-RPR1 cells were grown on tissue culture slides (Nunc, Naperville, IL) and were left untreated or were treated with 0.5 mM $CuSO_4$ for 90 min. Cells were washed once with PBS and were subsequently fixed in 2% glutaraldehyde in PBS for 10 min, after which they were washed three times with PBS. After a 30-min incubation at room temperature in PBS, 0.1% Triton X-100, 2% bovine serum albumin (BSA), and 1% normal goat serum, the cells were incubated for 2 hours with preimmune serum or with affinity-purified RPR antiserum in PBS, 0.1% Triton X-100, and 0.2% BSA. Cells were washed three times with PBS and 0.1% Triton X-100, and bound RPR antibodies were visualized by incubation with a goat antibody to rabbit immunoglobulin G coupled to fluorescein isothiocyanate.
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25. For ceramide quantitation, cells were treated as described (*16*). Lipids were extracted, and ceramide amounts were subsequently determined by in vitro phosphorylation in the presence of [γ-^{32}P]ATP (adenosine triphosphate) by diacyl-glyceride kinase relative to total cellular phospholipid [P. P. van Veldhoven, T. J. Matthews, D. P. Bolognesi, R. M. Bell, *Biochem. Biophys. Res. Commun.* **167**, 209 (1992)]. C2-ceramide and C6-ceramide (Gibco) were dissolved in ethanol at a concentration of 10 mM and diluted to a final concentration of 50 μM each in Grace's insect medium (Gibco) containing 0.5% FBS. The medium was sonicated in a bath sonicator for 5 s before being added to the cells. At different time points, the cells were stained with trypan blue, and the percent of positive cells was determined. The ethanol vehicle alone did not induce cell death.
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27. Single-letter abbreviations for the amino acid residues are as follows: C, Cys; G, Gly; H, His; K, Lys; P, Pro; R, Arg; S, Ser; T, Thr; and Y, Tyr.
28. We thank D. Schneider and J. Weiss for reagents and helpful discussions; and J. Escobedo, K. Giese, S. Harrison, M. Kavenaugh, and C. Reinhard for comments on the manuscript. G.J.P. was supported by a stipend from the Netherlands Organization for Scientific Research (NWO) during part of this work.

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Stimulation of Membrane Ruffling and MAP Kinase Activation by Distinct Effectors of RAS

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The RAS guanine nucleotide binding proteins activate multiple signaling events that regulate cell growth and differentiation. In quiescent fibroblasts, ectopic expression of activated H-RAS (H-RAS^{V12}, where V12 indicates valine-12) induces membrane ruffling, mitogen-activated protein (MAP) kinase activation, and stimulation of DNA synthesis. A mutant of activated H-RAS, H-RAS^{V12C40} (where C40 indicates cysteine-40), was identified that was defective for MAP kinase activation and stimulation of DNA synthesis, but retained the ability to induce membrane ruffling. Another mutant of activated H-RAS, H-RAS^{V12S35} (where S35 indicates serine-35), which activates MAP kinase, was defective for stimulation of membrane ruffling and induction of DNA synthesis. Expression of both mutants resulted in a stimulation of DNA synthesis that was comparable to that induced by H-RAS^{V12}. These results indicate that membrane ruffling and activation of MAP kinase represent distinct RAS effector pathways and that input from both pathways is required for the mitogenic activity of RAS.

RAS proteins are essential components of receptor-mediated signal transduction pathways that control cell proliferation and differentiation. RAS may control at least two signal transduction pathways, one regulating gene expression and the other controlling actin cytoskeleton organization (*1*, *2*). The first signaling pathway involves a series of cytoplasmic serine-threonine kinases, hereafter referred to as the MAP kinase pathway. The second pathway, hereafter referred to as the cell morphology pathway, is mediated by members of the Rho family of guanosine triphosphate (GTP) binding proteins, which regulate the organization of the actin cytoskeleton. The MAP kinase pathways and the cell morphology pathway can be dissociated (*3*, *4*). However, the point at which these pathways diverge has not yet been defined.

Morphological changes induced by activated forms of RAS proteins are accompanied by the induction of membrane ruf-

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