The Reaper (Rpr) gene encodes a 65-amino acid protein that induces apoptosis in Drosophila by an unknown mechanism. A previous study reported that Rpr expression induced generation of the lipid second messenger ceramide and through use of the peptide caspase inhibitors N-benzyloxycarbonyl-VAD-fluoromethylketone (zVAD.fmk) ordered ceramide generation downstream of caspases in SL2 cells (Pronk, G. J., Ramer, K., Amiri, P., and Williams, L. T. (1996) Science 271, 808–810). The present study re-evaluates these events in SL2 cells transfected with cDNA for Rpr, with or without the baculovirus caspase inhibitor p35, under the control of the metallothionein promoter. Following copper addition, Rpr protein was detected at 1.5 h and maximal at 2.5 h. Ceramide generation and caspase activation occurred nearly simultaneously, each detectable at 2–2.5 h and maximal at 6 h. Ceramide levels increased from a base line of 5 pmol/nmol lipid phosphorus to a maximum of 10 pmol/nmol lipid phosphorus. Identical increases in ceramide were detected using the enzymatic 1,2-diacetyl glycerol kinase assay or the non-enzymatic o-phthalaldehyde derivatization high pressure liquid chromatography assay. In contrast, diacylglycerol levels were not increased by Rpr expression. Apoptosis, first detected at 4 h, was maximal at 16 h. Co-expression of p35 did not affect Rpr-induced ceramide generation, whereas caspase activation and apoptosis were abolished. In contrast, zVAD.fmk inhibited ceramide generation and apoptosis. These data show that Rpr-induced ceramide generation is upstream or independent of p35-inhibitable caspases and demonstrate differences in the actions of peptide and p35 caspase inhibitors.

Apoptosis results from activation of a preprogrammed pathway of biochemical events that lead to cell death. One of the first genes to be identified from Drosophila as involved in apoptosis is the Reaper (Rpr) gene, which encodes a 65-amino acid protein. Deletion of a genomic region that contains the Rpr gene as well as two other pro-apoptotic genes, Grim and Head Involution Defective, blocked virtually all apoptosis that normally occurs during Drosophila embryogenesis (1–3). Overexpression of the Rpr gene in Drosophila retinas, in Drosophila embryos, and in Schneider line 2 (SL2) cells is sufficient to induce apoptosis (4–6). The mechanism by which Rpr induces apoptosis is still unclear. Rpr is hypothesized to be a death-inducing signal, as opposed to being part of the death effector machinery. It has been suggested that Rpr is the Drosophila homolog of the death domains of the human APO-1/Fas/CD95 and p55 tumor necrosis factor receptors (7), but this hypothesis was not supported by two mutagenesis studies on Rpr (8, 9).

The lipid molecule, ceramide, forms the backbone of all sphingolipids, including sphingomyelin and glycosphingolipids. Sphingomyelin hydrolysis by sphingomyelinases is induced in response to diverse stimuli, generating the second messenger ceramide. Such stimuli include the cytokines tumor necrosis factor-α, interleukin-1β, and nerve growth factor, ligands for the CD95 and CD28 receptors, and cellular stresses such as ionizing radiation and ultraviolet light, to list a few (10–12). A second pathway for ceramide generation involving the enzyme ceramide synthase is activated by the chemotherapeutic agent, daunorubicin (13). A number of direct targets for ceramide action have been identified, including a ceramide-activated protein kinase (14, 15), a ceramide-activated protein phosphatase (16), and the protein kinase Cζ isoform (17). Ceramide also indirectly activates signaling cascades involving stress-activated protein kinase/Jun N-terminal kinase (18), p38 (19), and p42/p44 mitogen-activated protein kinases (20). Ceramide signals apoptosis, differentiation, or growth arrest, depending on the cell type and context in which ceramide is generated. In this regard, ceramide is generated during the induction of apoptosis in a wide variety of cellular systems (10, 21). Addition of cell-permeable ceramide analogs, but not analogs of other lipid second messengers, induces apoptosis in many of these cell lines, providing evidence that ceramide generation plays a direct role in the apoptotic response. Furthermore, genetic models of sphingomyelinase deficiency that lack ceramide generation display reductions in apoptosis upon exposure to stress. Cells from patients with Niemann-Pick disease fail to respond to ionizing radiation with ceramide generation or apoptosis, and retroviral transfer of the acid sphingomyelinase gene restores both events (22). Similarly, acid sphingomyelinase knock-out mice present a deficit in radiation-induced ceramide generation and endothelial apoptosis in the lung but no defect in p53-mediated death of thymocytes (22). Additional support for a requirement for ceramide generation is derived from glycerol; zVAD.fmk, N-benzyloxycarbonyl-VAD-fluoromethylketone; FB1, fumonisin B1; CPE, ceramide phosphorylthanolamine.

Ron Bose‡‡, Po Chen¶¶, Andrea Loconti‡, Carsten Grullich‡, John M. Abrams§§, and Richard N. Kolesnick‡‡‡

From the ‡‡‡Laboratory of Signal Transduction, Memorial Sloan-Kettering Cancer Center, New York, New York 10021 and the §§‡Department of Cell Biology and Neuroscience, University of Texas Southernmost Medical Center, Dallas, Texas 75235

* This work was supported in part by Grant AG12466 (to J. M. A.) from the National Institutes of Health and Grant CB-80362 from the American Cancer Society. The costs of publication of this article were from the National Institutes of Health.

** Supported by National Institutes of Health MSTP Grant GM07739 and the Lee Friedman Memorial Fellowship.

† Supported by Postdoctoral Fellowship GM18215 from the National Institutes of Health.

The abbreviations used are: Rpr, Reaper; SL2, Schneider line 2; ECL, enhanced chemiluminescence; HPLC, high pressure liquid chromatography; OPA, o-phthalaldehyde; HA, hemagglutinin; MeSO2, dimethyl sulfoxide; PARP, poly(ADP-ribose) polymerase; DAG, diacylglycerol; zVAD.fmk, N-benzyloxycarbonyl-VAD-fluoromethylketone; FB1, fumonisin B1; CPE, ceramide phosphorylthanolamine.

Printed in U.S.A. © 1998 by The American Society for Biochemistry and Molecular Biology, Inc.

American Cancer Society. The costs of publication of this article were from the National Institutes of Health and Grant CB-80362 from the American Cancer Society.

Ceramide Generation by the Reaper Protein Is Not Blocked by the Caspase Inhibitor, p35*

(Received for publication, February 26, 1998, and in revised form, July 6, 1998)
studies using daunorubicin in P388 lymphoma cells. In this system, daunorubicin induces prolonged ceramide generation by activation of ceramide synthase, and the mycotoxin fumonisin B1, a specific inhibitor of ceramide synthase, blocks ceramide generation and apoptosis (13). Fumonisin B1 also abrogated daunorubicin-induced apoptosis in hen granulosa cells (23) and CPT-11-induced apoptosis in L929 cells (24). In sum, ceramide appears to be a generic signal for the apoptotic response.

Caspases are a family of cysteine proteases, formerly known as the ICE/Ced-3 family, that specifically cleave proteins after aspartate residues. The critical role of caspases in apoptosis was first demonstrated by the isolation of the cd-3 gene by genetic screens for cell death-defective mutants in Caenorhabditis elegans (25). An abundance of evidence supports the notion that caspases are the effectors of apoptosis in mammalian systems. Currently, at least 10 mammalian caspases and 2 Drosophila caspases have been cloned (26–30). Physiologic caspase inhibitors, such as the cowpox virus CrmA protein and the baculovirus p35 protein, and peptide caspase inhibitors designed to compete for the substrate recognition site (27) have been used effectively to block stress-induced apoptosis.

Numerous studies have addressed the role of caspases in ceramide-induced apoptosis. Ceramide has been reported to function upstream of caspase-3 or caspase-3-like proteases during induction of apoptosis (31–38); however, the relationship to caspase activation and apoptosis. Thus, these two caspase inhibitors, which are stream or parallel to p35-inhibitable caspases. Additionally, we examined this result using cells transfected with the baculovirus p35 protein under the control of a metallothionein promoter. We demonstrate that ceramide is generated in SL2 cells rapidly following induction of the Rpr gene. Ceramide generation and caspase activation are nearly simultaneous and precede apoptosis. Cells co-expressing Rpr and p35 manifest a ceramide rise despite blockade of caspase activation and apoptosis, demonstrating that ceramide elevation is either upstream or parallel to p35-inhibitable caspases. Additionally, we have confirmed that zVAD.fmk inhibits ceramide generation and apoptosis. Thus, these two caspase inhibitors, which are generally regarded as having similar broad specificities, may have very different effects in some systems (27, 40). This report is the first to demonstrate a difference between peptide and p35 caspase inhibitors and encourages caution when interpreting the results of caspase inhibitor studies.

EXPERIMENTAL PROCEDURES

Materials and Equipment—Fumagillin B, gentamicin, ceramide type III, sphingomyelin, galactosylceramide, glucosylceramide, and fumonisin B1 were purchased from Sigma. SF900 II serum-free media supplemented with 50 μg/ml gentamicin, and transfected SL2 cells additionally received 300 μg/ml hygromycin B (8). The construction of transfected SL2 cells was described previously (5, 8). The Rpr and Rpr2 lines are two independent transfections of the same metallothionein promoter-HA epitope-Rpr open reading frame construct. The Rpr + p35 line was made by simultaneous transfection of metallothionein promoter-HA epitope-Rpr open reading frame and metallothionein promoter-p35 cDNA constructs. All three transfectants represent pooled populations of resulting hygromycin-resistant cells. The Rpr line displayed the strongest degree of Rpr expression, as judged by immunoblot (see below) and by immunofluorescence, and it is estimated that approximately 50% of all cells in this line express Rpr protein following copper addition (5).

Ceramide was measured at 25 °C and split to an initial density of 1 × 10⁶ cells/ml every 5 to 6 days. Four days prior to experiments, cells were trypsinized, centrifuged, and resuspended in serum-free media supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin.

The Rpr line displayed the strongest degree of Rpr expression, as judged by immunoblot (see below) and by immunofluorescence, and it is estimated that approximately 50% of all cells in this line express Rpr protein following copper addition (5).

Measurement of ceramide was performed using 3 ml of CHCl₃/CH₃OH/1 N HCl (100:100:1). The organic phase was dried down under N₂ and 0.5 ml of 1 M KOH and 0.5 ml of 1M HCl in CH₃OH, 1.0 ml of CHCl₃, and 0.75 ml of H₂O were added to each tube. The tubes were heated at 90 °C for 1 hour to convert quantitatively ceramide into sphingosine. This digestion procedure did not convert complex sphingolipids, such as sphingomyelin, galactosylceramide, or glucosylceramide, into sphingosine. Following digestion, samples and standards were extracted with 0.5 ml of 1 N HCl in CH₃OH, 1.0 ml of CHCl₃, and 0.75 ml of 1 M aqueous NaCl. The organic phase was dried under N₂ and redissolved in 0.1 ml of CH₃OH. A solution of OPA was prepared fresh daily by dissolving 10 mg of OPA in 0.2 ml of ethanol and then adding

FIG. 1. Time course of Rpr expression following transgene induction by copper. Cells stably transfected with HA-tagged Rpr under the control of the metallothionein promoter were treated with 0.7 mm copper sulfate for the indicated times. The cells were lysed in Laemml sample buffer, and an immunoblot was performed using anti-HA antibodies. Data are representative of five experiments.

420/420AC Fluorescence Detector, and model 746 Data Module Integrator.

Cell Culture—Schneider line 2 (SL2) cells (41) were grown in SF900 II serum-free media supplemented with 50 μg/ml gentamicin, and transfected SL2 cells additionally received 300 μg/ml hygromycin B (8).

The construction of transfected SL2 cells was described previously (5, 8). The Rpr and Rpr2 lines are two independent transfections of the same metallothionein promoter-HA epitope-Rpr open reading frame construct. The Rpr + p35 line was made by simultaneous transfection of metallothionein promoter-HA epitope-Rpr open reading frame and metallothionein promoter-p35 cDNA constructs. All three transfectants represent pooled populations of resulting hygromycin-resistant cells. The Rpr line displayed the strongest degree of Rpr expression, as judged by immunoblot (see below) and by immunofluorescence, and it is estimated that approximately 50% of all cells in this line express Rpr protein following copper addition (5).

Ceramide was measured at 25 °C and split to an initial density of 1 × 10⁶ cells/ml every 5 to 6 days. Four days prior to experiments, cells were trypsinized, centrifuged, and resuspended in serum-free media supplemented with 100 units/ml penicillin and 0.1 mg/ml streptomycin. Cell density was determined by hemacytometer and the percentage of dead cells by trypan blue exclusion analysis. For induction of the transgene from the metallothionein promoter, cells were exposed to 0.7 mm copper sulfate. All experiments were performed with cells in log phase growth at a density of 1.5–3.0 × 10⁶/ml.

C₂₀-sphingosine and C₂₀-dihydroceramide stock solutions were prepared in dimethyl sulfoxide (MeSO) and 100% ethanol, respectively. zVAD.fmk and zDEVD.fmk stock solutions were prepared in MeSO. The final concentration of these solvents in the incubations was 0.2%, which did not induce apoptosis or affect cell viability. Fumonisin B1 was dissolved in sterile water.

Immunoblot Analysis of HA-Rpr—At various times after copper sulfate treatment, cells were pelleted by centrifugation (2000 × g, 5 min) and solubilized in Laemml sample buffer containing 2.5% 2-mercaptoethanol (42). Whole cell lysates from 5 × 10⁵ cells were resuspended in 15% SDS-polycrylamide gel electrophoresis and transferred to nitrocellulose membranes. The blots were probed with anti-hemagglutinin (HA) monoclonal antibody and visualized by ECL. Densitometry was performed using a Macintosh IIX computer, desktop scanner, and Scan Analysis software (43).

Lipid Studies—Measurement of ceramide and DAG by the DAG kinase assay was performed as described previously (13). SL2 cell lipids were prepared by extracting 0.5–2.0 × 10⁶ cells with 1.5 ml of CHCl₃/CH₃OH/H₂O 1:1:1 (HCl 100:100:1, v/v/v). Standard curves were generated either with 50–1,000 pmol of ceramide type III or with 40–2,500 pmol of DAG. It is important to note that under the assay conditions described above, the reaction goes to completion and hence is not subject to activators contained within a biologic sample (44).

Measurement of ceramide by HPLC fluorescence spectrometry was performed as a modification of the procedure described by Merrill and co-workers (45). For these studies, standards of 100–2,000 pmol ceramide type III were prepared. Approximately 3 × 10⁵ cells were extracted using 3 ml of CHCl₃/CH₃OH/0.1 M HCl (100:100:1). The organic phase was dried under N₂ and then re-extracted. An internal standard of 500 pmol of a 20-carbon analog of sphingosine was added to both samples and standards. All tubes were dried down under N₂ and 0.5 ml of 1 N KOH in 90% CH₃OH was added. Tubes were sealed with screw caps and heated at 90 °C for 1 h to convert quantitatively ceramide into sphingosine. After digestion procedure did not convert complex sphingolipids, such as sphingomyelin, galactosylceramide, or glucosylceramide, into sphingosine. Following digestion, samples and standards were extracted with 0.5 ml of 1 M HCl in CH₃OH, 1.0 ml of CHCl₃, and 0.75 ml of 1 M aqueous NaCl. The organic phase was dried under N₂ and redissolved in 0.1 ml of CH₃OH. A solution of OPA was prepared fresh daily by dissolving 10 mg of OPA in 0.2 ml of ethanol and then adding
10 μl of 2-mercaptoethanol and 19.8 ml of 3% aqueous boric acid (pH adjusted to 10.5 with KOH). 0.1 ml of the OPA solution was added to the tubes, and they were incubated at 22 °C for 5 min. 0.5 ml of solvent A (CH₃OH, 5 mM aqueous potassium phosphate, pH 7.0 (90:10, v/v)) was added, and samples and standards were resolved by reverse phase HPLC using a Nova Pak C18 column with isocratic elution using solvent A. The following settings were employed: injection volume, 20 μl; solvent flow rate, 0.6 ml/min; cycle time, 60 min; fluorescence excitation wavelength, 340 nm; and emission wavelength, 455 nm. Free sphingosine was measured by use of milder digestion conditions, 0.1M KOH in CH₃OH at 37 °C for 1 h, with subsequent steps identical to those above. Retention times of various sphingosines depended on alkyl chain length: the 14-, 16-, 18-, and 20-carbon analogs of sphingosine eluted in 3.6, 5.3, 8.1, and 13.7 min, respectively. Formally, the fluorescence signal represents the sum of ceramide and free sphingosine, but the contribution of free sphingosine is minor as ceramide levels are 10–20-fold greater than free sphingosine.

Measurement of ceramide via metabolic labeling was performed as follows (46): 1×10⁶ cells were labeled with 50 μCi of [9,10-3H]palmitic acid (56 Ci/mmol) for 48 h. Following treatment with 0.7 mM copper sulfate, cells were dissolved in 0.5 ml of CH₃OH and extracted with 0.5 ml of H₂O and 1.0 ml of CHCl₃. The organic phase was dried under N₂ and re-dissolved in CHCl₃. Equal amounts of radioactive material (5×10⁶ cpm) were resolved by TLC in a solvent system of ethyl ether: benzene:ethanol:acetic acid (40:50:2.2:0.2, v/v). TLC plates were sprayed with En³hance prior to autoradiography. The migration of ceramide standards was determined by iodine staining, and the amount of co-migrating radioactivity was quantified by liquid scintillation counting.

**Fig. 2.** Measurement of ceramide levels, by two independent methods, and diacylglycerol levels following induction of Rpr by copper. A, cells stably transfected with Rpr and wild-type SL2 cells were treated with 0.7 mM copper sulfate for the indicated time, to induce Rpr expression, and then extracted to obtain cellular lipids. Ceramide content was measured via the DAG kinase assay and normalized for total lipid phosphorus. Base-line ceramide mass was 5.7 and 5.5 pmol of ceramide per nmol of lipid phosphorus for Rpr transfectants and wild-type cells, respectively. Each value represents mean ± S.E. of triplicate determinations from six experiments. B, cells were treated as in A, and ceramide levels were measured by HPLC fluorescence spectrometry after derivatization with OPA. By using this method, base-line ceramide mass was measured as 4.5 and 4.4 pmol of ceramide per nmol of lipid phosphorus for Rpr transfectants and wild-type cells, respectively. Data represent mean ± range of duplicate determinations from one of two similar experiments. C, cells were treated as in A, and DAG content was measured using the DAG kinase assay. Base-line DAG mass was 23 and 21 pmol of DAG per nmol of lipid phosphorus for Rpr transfectants and wild-type cells, respectively. Each value represents mean ± S.E. of triplicate determinations from two experiments.
Quantitation of total lipid phosphorus was performed by digesting the lipid extracts from cells to release inorganic phosphate, which was then quantified by a microscale phosphorus assay (47). Briefly, cells were extracted in manner identical to that used for the DAG kinase assay. The dried lipid extracts were re-extracted using 0.5 ml of CHCl3, 0.5 ml of CH3OH, and 0.3 ml of buffered saline solution (135 mm NaCl, 1.5 mm CaCl2, 0.5 mm MgCl2, 5.6 mm glucose, and 10 mm HEPES, pH 7.2). 0.1 ml of 10% magnesium nitrate in ethanol was added to the resulting organic phase, and samples were dried by heating at 80 °C and flamed using a Bunsen burner for several seconds. 0.3 ml of 0.5 N HCl was added, and tubes were heated to boiling for 15 min. 0.6 ml of 0.42% ammonium molybdate in 1.0 N sulfuric acid and 0.1 ml of 10% ascorbic acid solution were added and tubes were incubated at 45 °C for 30 min. Absorbance was read at 820 nm and phosphorus content determined by comparison to standard curve obtained with 2–15 nmol of monosodium phosphate.

In Vitro Caspase Assay—At various times after copper sulfate treatment, 3–6 × 106 cells were collected by centrifugation (2000 × g, 10 min, 4 °C), washed once with ice-cold SF900 II serum-free media, resuspended in 0.1 ml of lysis buffer (25 mm HEPES, pH 7.5, 10 mm KCl, 1.5 mm MgCl2, 1.0 mm EDTA, 1.0 mm EGTA, 1.0 mm dithiothreitol, 0.1 mm phenylmethylsulfonyl fluoride, 5 μg/ml pepstatin, 10 μg/ml leupeptin, and 2 μg/ml aprotinin), and homogenized on ice using a motor-driven, microcentrifuge tube pestle. Samples were centrifuged (4000 × g, 10 min, 4 °C), and the protein concentration in the resulting supernatant was determined by the method of Bradford (48) using bovine serum albumin standards. A reaction containing 50 μg of supernatant protein and 100 ng of partially purified bovine PARP protein in a total volume of 20 μl was incubated for 1 h at 25 °C. The reaction was stopped by adding Laemmli sample buffer containing 2.5% 2-mercaptoethanol. Samples were resolved on 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, probed with anti-PARP monoclonal antibody, and visualized using ECL.

Flow Cytometry—Analysis of DNA content of SL2 cells was performed as described previously in (5). Briefly, cells were fixed in 2% formaldehyde in phosphate-buffered saline for 15 min, stained with 50 μg/ml propidium iodide, and analyzed within 24 h on a Becton-Dickinson FACScan flow cytometry using LYSIS II software.

RESULTS

Expression of Rpr in Drosophila embryos and in SL2 cells is sufficient for induction of apoptosis (1, 6, 8). To study the biochemical signals involved in Rpr-induced apoptosis, we used SL2 cells, a cell line originally derived from Drosophila embryos, stably transfected with the Rpr open reading frame driven, microcentrifuge tube pestle. Samples were centrifuged (4000 × g, 10 min, 4 °C), and the protein concentration in the resulting supernatant was determined by the method of Bradford (48) using bovine serum albumin standards. A reaction containing 50 μg of supernatant protein and 100 ng of partially purified bovine PARP protein in a total volume of 20 μl was incubated for 1 h at 25 °C. The reaction was stopped by adding Laemmli sample buffer containing 2.5% 2-mercaptoethanol. Samples were resolved on 7.5% SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes, probed with anti-PARP monoclonal antibody, and visualized using ECL.

Flow Cytometry—Analysis of DNA content of SL2 cells was performed as described previously in (5). Briefly, cells were fixed in 2% formaldehyde in phosphate-buffered saline for 15 min, stained with 50 μg/ml propidium iodide, and analyzed within 24 h on a Becton-Dickinson FACScan flow cytometry using LYSIS II software.

TABLE I

Comparison of time courses in Rpr cells following addition of copper

| Initially detected Maximal |
|---------------------------|
| h                        |
| HA-Reaper expression      | 1–1.5 | 2.5  |
| Ceramide elevation        | 2–2.5 | 6    |
| Caspase activation        | 2–2.5 | 6    |
| Apoptosis                 | 4     | 16   |

To investigate whether ceramide is involved in Rpr action, ceramide levels were measured by three independent methodologies following Rpr induction. In Fig. 2A, ceramide levels were measured by the DAG kinase assay. Ceramide levels in Rpr transfectants began to increase at 2–2.5 h after copper addition from a base-line level of 5.5 pmol of ceramide/nmol of lipid phosphorus and reached 186% of control at 5 h. In two experiments, time courses were extended out to 12 h, and ceramide levels were found to reach a peak of 208% of control at 6 h and remain elevated at 203% of control for 12 h after copper addition (data not shown). Ceramide levels in wild-type SL2 cells did not change following copper addition.

Recently, the validity of the DAG kinase assay to measure ceramide levels has been questioned (49). To confirm the ceramide elevations detected with the DAG kinase assay after Rpr induction, ceramide levels were measured by two non-enzymatic techniques as follows: by HPLC fluorescence spectrometry after derivatization with OPA, and by metabolic labeling with [3H]palmitic acid. As seen in Fig. 2B, the magnitude and time course of the ceramide rise in the Rpr expressing cells as determined by the HPLC fluorescence spectrometry assay were virtually identical to that obtained by the DAG kinase assay. Similarly, no elevation in ceramide levels was detected in wild-type SL2 cells after copper treatment using HPLC fluorescence spectrometry (Fig. 2, A and B).

Ceramide levels were also measured using a metabolic labeling approach using cells labeled to isotopic equilibrium with [3H]palmitic acid. By this method, cells expressing Rpr showed a 71% increase in ceramide at 6 h after copper addition (data not shown). Control cells transfected with empty vector displayed no increase in ceramide at 6 h. Thus metabolic labeling with [3H]palmitic acid confirmed the specific elevation in ceramide levels detected by the DAG kinase and HPLC fluorescence spectrometry methods.

DAG levels were also determined in response to expression of Rpr. In contrast to ceramide, expression of Rpr did not elevate DAG levels. In fact, copper treatment induced a small, 15–20% decrease in DAG levels in both wild-type and Rpr expressing SL2 cells (Fig. 2C). Thus, the effect of Rpr to generate an increase in the level of ceramide was specific.
Previous reports implicated caspases in Rpr-induced apoptosis since caspase inhibitors blocked Rpr-induced apoptosis (4–6). We sought to assay caspases and determine the rapidity of their activation in this system by use of an in vitro protease assay. Rpr transfectants were cultured for various periods in the presence of copper sulfate, after which caspase activity in cellular lysates was determined by using bovine poly(ADP-ribose) polymerase (PARP) as substrate. Since mammalian PARP is a good substrate for at least two invertebrate caspases, DCP-1 from Drosophila and ced-3 from C. elegans (28, 52), bovine PARP was selected as substrate for the caspase assay using SL2 cell lysates. Cleavage of the full-length, 116-kDa form of PARP to the expected 85-kDa fragment indicative of caspase activation was first detected in lysates from Rpr-transfected cells at 2–2.5 h after copper addition (Fig. 3). Maximal caspase activity was seen at 6 h, and by 12 h there was a slight decrease in caspase activity. No caspase activity was detected in wild-type cells in the absence or presence of copper (data not shown). The immunoblotting has a limit of detection of 3 ng of PARP, and therefore this assay can detect cleavage of as little as 3% of the total amount of substrate added. These studies demonstrate that Rpr-induced apoptosis is accompanied by ceramide generation and caspase activation.

Table I summarizes the time courses of Rpr expression, ceramide generation, caspase activation, and induction of apoptosis. As expected, Rpr protein expression was the most rapid of these four events following copper addition. Subsequently, ceramide levels increased and caspases became activated. These events were nearly simultaneous, being initially detectable at 2–2.5 h and maximal at 6 h after copper addition. Finally, apoptosis was detected by flow cytometry as early as 4 h and peaked at 16 h. Based on these data, it is clear that both ceramide generation and caspase activation preceded apoptosis. However, the relative order of ceramide generation and caspase activation cannot be ascertained from this information. To molecularly order these two events, we used baculovirus p35 a known inhibitor of caspases (53).

Expression of p35 has been shown to inhibit Rpr-induced cell death in both SL2 cells and in transgenic Drosophila (4, 5). As described above, co-expression of p35 blocked Rpr-induced apoptosis. For the subsequent analysis, the Rpr + p35 cell line was compared with the Rpr2 cell line, a second, independent Rpr transfectant derived with the same construct as the original Rpr line. Both the Rpr + p35 and Rpr2 cell lines expressed comparable levels of Rpr which was somewhat less than the Rpr-transfected cell line used for the above studies, as determined by immunoblotting (data not shown). The in vitro caspase assay using bovine PARP as substrate was performed on Rpr + p35 and Rpr2 cells (Fig. 4A). No caspase activity was detected by this assay in the Rpr + p35 cells after gene induction by copper, whereas abundant caspase activity was seen in the Rpr2 cell line. Thus it appears that p35 prevented Rpr-mediated caspase activation. Measurements of the ceramide level in Rpr + p35 cells following gene induction by copper (Fig. 4B) demonstrated that a ceramide rise still occurs and in fact was slightly greater in these cells, despite complete blockade of apoptosis and caspase activity. Cells that were stably transfected with only the p35 gene showed no changes in ceramide level following gene induction by copper (data not shown). These data strongly suggest that ceramide elevation is either upstream or parallel to p35-inhibitable caspases.

In contrast to p35, the peptide caspase inhibitor, zVAD.fmk, was reported to inhibit both Rpr-mediated ceramide generation and apoptosis (6). In the present investigation, we re-examined...
this issue. zVAD.fmk had no effect on Rpr expression or baseline ceramide levels (data not shown). However, Rpr-induced apoptosis, as measured by cells with hypodiploid DNA content at 18 h after copper addition, was completely inhibited by 10 μM zVAD.fmk; an IC_{50} for this effect was approximately 5 μM zVAD.fmk (Fig. 5A). Ceramide generation, measured 4 h after copper addition, was similarly inhibited by zVAD.fmk. Complete inhibition of Rpr-induced ceramide generation occurred with 10 μM zVAD.fmk, and an IC_{50} was approximately 3 μM zVAD.fmk (Fig. 5B). Treatment with zVAD.fmk even 1.5 to 2.5 h after copper addition, a time at which Rpr is already expressed, blocked subsequent ceramide generation (data not shown). zDEVD.fmk, at 25 μM, similarly abolished Rpr-induced ceramide generation and apoptosis whereas up to 100 μM DEVD.CH0, which is not cell-permeable, was without effect. Thus, our investigations reproduce the data published by Pronk et al. (6). These studies show that the peptide caspase inhibitor zVAD.fmk and the viral caspase inhibitor p35, both of which are generally regarded as having broad specificity, may have different effects in some systems.

**DISCUSSION**

The present study demonstrates that Rpr-induced ceramide elevation is not blocked by the caspase inhibitor, p35. Ceramide generation was measured by three independent techniques, verifying the previously published report of Pronk and co-workers (6). In contrast, DAG levels did not rise, indicating ceramide is generated specifically in response to Rpr expression. Detailed time courses of the early events after Rpr induction revealed that ceramide generation and caspase activation occur rapidly, were nearly simultaneous events, and preceded morphological and nuclear manifestations of apoptosis. Thus ceramide generation is not a late event in the apoptotic response to Rpr. As anticipated, co-expression of p35 blocked Rpr-induced caspase activation and apoptosis. However, p35 did not affect Rpr-induced ceramide generation. Based on these results, we exclude the possibility that ceramide generation is a consequence of activation of p35-inhibitable caspases or the induction of apoptosis in response to Rpr.

In the present investigations, we have also verified the published data of Pronk and co-workers (6) on the effects of zVAD.fmk. We observed that zVAD.fmk blocked both ceramide generation and apoptosis following Rpr induction. The ability of zVAD.fmk to block ceramide generation suggests two possibilities. Either there exists a zVAD.fmk-sensitive, p35-insensitive caspase whose activation is required for ceramide generation or zVAD.fmk may inhibit other targets in addition to caspases. Currently, this issue cannot be resolved. However, if a zVAD.fmk-sensitive, p35-insensitive caspase exists, it would appear incapable of cleaving mammalian PARP and would be insufficient in and of itself to signal Rpr-induced death.

Recent studies (28, 29) provide information regarding individual caspases of *Drosophila*. Two *Drosophila* caspases, DCP-1 and drICE, have been cloned, and several more unidentified caspases may exist (28, 29). Both have greater homology to mammalian caspase-3 than to caspase-1 and are suggested to have DXXD substrate specificity. Processing of drICE was demonstrated in SL2 cells undergoing Rpr-induced apoptosis and drICE was argued to be one of the major caspases present in SL2 cells (29, 55). Recently, Nagata and colleagues (56) biochemically characterized caspases activated during Rpr-induced apoptosis in SL2 cells. Both caspase-3-like DEVDase and caspase-1-like YVADase activity displayed similar time courses of activation, with peak activity at 6 h. There was no evidence for an early caspase-1-like activity preceding the caspase-3-like activity in this system. Caspase activity was fractionated into two distinct peaks on DEAE column chromatography; the first peak contained only DEVDase activity, and the second had both DEVDase and YVADase activity. zVAD.fmk inhibited only the first peak, whereas both peaks were sensitive to DEVD-aldehyde (56). Both peaks would presumably be sensitive to p35 as well since p35 contains a DQMD site which is essential for caspase inhibition (57). Thus, the published studies provide no support for a zVAD.fmk-sensitive, p35-insensitive caspase activity during Rpr-induced apoptosis.
Although zVAD.fmk and p35 are generally regarded to be broad range caspase inhibitors, a comparison of their relative specificities for individual caspases has not been performed. Livingston and colleagues (58) point out that irreversible peptide caspase inhibitors at concentrations of 20 µM or greater, and for reaction times greater than 1 h, as commonly used for whole cell studies of apoptosis, can be expected to show little discrimination among members of the caspase family. Similarly, p35 appears to inhibit mammalian caspases 1–4 and the C. elegans caspase, ced-3, with equal potency (53). Little information is currently available as to whether viral or peptide caspase inhibitors affect cellular targets other than caspases. In this regard, expression of p35 in Drosophila retinas resulted in eyes with little or no abnormalities, suggesting that p35 has minimal, if any, nonspecific effects (2, 4, 59). In contrast, irreversible inhibitors containing halomethylketones, such as the fmk group, are fairly reactive and at 10 µM covalently label multiple proteins in lysates. Hence, nonspecific effects of halomethylketone caspase inhibitors may contribute to the differences observed between p35 and zVAD.fmk with respect to ceramide generation.

Additional issues remain unresolved regarding the role of ceramide in Rpr action. Neither the source of the ceramide generated nor its role in apoptosis are yet defined. During signaling in mammalian systems, ceramide is generated either by hydrolysis of sphingomyelin, mediated by sphingomyelinases, or, by de novo synthesis, mediated by ceramide synthase. Dipteran flies were previously noted to contain virtually no sphingomyelin but instead have ceramide phosphoryl ethanolamine (CPE), an analogous sphingolipid with a phosphoethanolamine head group rather than the phosphocholine found on sphingomyelin (60, 61). In preliminary studies, we found that SL2 cells contain approximately 600 pmol of CPE/10^6 cells and undetectable levels of sphingomyelin. No consistent alterations in CPE levels following Rpr induction were detected, suggesting that CPE is not the source of ceramide (data not shown). Furthermore, we have been unable to measure ceramide synthase activity in SL2 cells under conditions optimal for the mammalian enzyme, suggesting that Drosophila ceramide synthase requires different assay conditions (data not shown). Nevertheless, fumonisin B1, a mycotoxin which inhibits ceramide synthase requires different assay conditions (data not shown). Furthermore, we have been unable to measure ceramide synthase activity in SL2 cells under conditions optimal for the mammalian enzyme, suggesting that Drosophila ceramide synthase requires different assay conditions (data not shown). Nevertheless, fumonisin B1, a mycotoxin which inhibits ceramide synthase requires different assay conditions (data not shown). Furthermore, we have been unable to measure ceramide synthase activity in SL2 cells under conditions optimal for the mammalian enzyme, suggesting that Drosophila ceramide synthase requires different assay conditions (data not shown). Nevertheless, fumonisin B1, a mycotoxin which inhibits ceramide synthase requires different assay conditions (data not shown). Furthermore, we have been unable to measure ceramide synthase activity in SL2 cells under conditions optimal for the mammalian enzyme, suggesting that Drosophila ceramide synthase requires different assay conditions.
46. Liu, P., and Anderson, R. G. W. (1995) J. Biol. Chem. 270, 27179–27185
47. Chen, J. P. S., Torihara, T. Y., and Warner, H. (1956) Anal. Chem. 28, 1756–1758
48. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
49. Watts, J. D., Gu, M., Polverino, A. J., Patterson, S. D., and Aebersold, R. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 7292–7296
50. Deleted in proof
51. Deleted in proof
52. Xue, D., Shaham, S., and Horvitz, H. R. (1996) Genes Dev. 10, 1073–1083
53. Bump, N. J., Hackett, M., Hugunin, M., Seshagiri, S., Brady, K., Chen, P., Ferenz, C., Franklin, S., Ghayur, T., Li, P., Licari, P., Mankovich, J., Shi, L., Greenberg, A. H., Miller, L. K., and Wong, W. W. (1995) Science 269, 1885–1888
54. Deleted in proof
55. Fraser, A. G., McCarthy, N. J., and Evan, G. I. (1997) EMBO J. 16, 6192–6199
56. Kondo, T., Yokokura, T., and Nagataki, S. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 11831–11836
57. Xue, D., and Horvitz, H. R. (1995) Nature 377, 248–251
58. Margolin, N., Raybuck, S. A., Wilson, K. P., Chen, W., Fox, T., Gu, Y., and Livingston, D. J. (1997) J. Biol. Chem. 272, 7223–7228
59. Hay, B. A., Wolff, T., and Rubin, G. M. (1994) Development 120, 2121–2129
60. Stark, W. A., Lin, T. N., Brackhahn, D., Christianson, J. S., and Sun, G. Y. (1993) Lipids 28, 23–28
61. Laukkonen, A., Kuusniemi, L., and Renkonen, O. (1976) Biochim. Biophys. Acta 450, 109–120
62. Merrill, A. H., van Echten, G., Wang, E., and Sandhoff, K. (1993) J. Biol. Chem. 268, 27299–27306
63. Wu, W.-I., McDonough, V. M., Nickels, J. T., Jr., Ko, J., Fischl, A. S., Vales, T. R., Merrill, A. H., Jr., and Carman, G. M. (1995) J. Biol. Chem. 270, 13171–13178
64. Fishbein, J. D., Dobrowsky, R. T., Bielawska, A., Garrett, S., and Hannun, Y. A. (1993) J. Biol. Chem. 268, 9255–9261
65. Dickson, R. C., Nagiec, E. E., Skrzypek, M., Tillman, P., Wells, G. B., and Lester, R. L. (1997) J. Biol. Chem. 272, 30196–30200