Role of *CAS*, a Human Homologue to the Yeast Chromosome Segregation Gene *CSE1*, in Toxin and Tumor Necrosis Factor Mediated Apoptosis

Ulrich Brinkmann,* Elisabeth Brinkmann, Maria Gallo, Uwe Scherf, and Ira Pastan

Laboratory of Molecular Biology, DBS, National Cancer Institute, National Institutes of Health, Building 37, Room 4E16, 37 Convent Drive MSC 4255, Bethesda, Maryland 20892-4255

Received November 29, 1995; Revised Manuscript Received March 11, 1996[⊗]

ABSTRACT: We have previously isolated by expression/selection cloning plasmids containing human cDNAs that rendered MCF-7 breast cancer cells resistant to immunotoxins, Pseudomonas exotoxin (PE), and diphtheria toxin (DT) [Brinkmann *et al*. (1995) *Mol. Med. 1*, 206−216]. Here we describe that one of these resistant plasmids, which contains an antisense cDNA fragment homologous to the yeast chromosome segregation gene *CSE1* [*CAS*; Brinkmann et al. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10427−10431], reduces the intracellular content of the human CSE1 homologue CAS protein. CAS reduction confers resistance not only to the ADP-ribosylating toxins PE and DT, but also to tumor necrosis factor α and *β*. The resistance was observed as reduced apoptosis. CAS antisense did not affect the cell death induced by staurosporine, cycloheximide, or etoposide. The observation that CAS antisense can interfere with apoptosis mediated by TNF and ADP-ribosylating toxins suggests that *CAS* may play a role in selected pathways of apoptosis.

Apoptosis is a carefully regulated network of biochemical events which lead to cell death. Apoptosis can be triggered in various ways, including virus infection, growth factor withdrawal, DNA damage, or by signals such as TNF[1] binding to its receptor or by cross-linking the Fas receptor with anti-Fas antibodies (Cohen, 1993; Lowe et al., 1993; Sentman et al., 1991; Smith et al., 1994; Suda et al., 1993; Williams & Smith, 1993). Although the pathways leading to apoptosis are not fully elucidated, several genes that play a role in the process have been identified, and some, such as *P53* or *BCL-2*, have an important role in cancer (Lowe et al., 1993; Sentman et al., 1991). One way of inducing apoptosis is exposure of cells to diphtheria toxin (DT) or Pseudomonas toxin (PE) (Chang et al., 1989; Kochi & Collier, 1993; Morimoto & Bonavida, 1992); both of these bacterial toxins inhibit eukaryotic protein synthesis by ADP-ribosylating and thereby inactivating elongation factor 2 (Carroll & Collier, 1987). It is not clear how these toxins induce apoptosis, but it is a toxin-specific mechanism and not simply a general reaction to inhibition of protein synthesis (Chang et al., 1989; Morimoto & Bonavida, 1992).

Immunotoxins containing recombinant forms of PE and DT specifically target and kill cancer cells (Brinkmann & Pastan, 1994; Brinkmann et al., 1991; Pastan et al., 1992). Several immunotoxins are currently being evaluated in clinical trials (Brinkmann & Pastan, 1994). If immunotoxins eventually have a useful role in cancer treatment, one must be aware that all drugs that are used for cancer treatment can lose their efficacy due to the development of drug resistance (Gottesman & Pastan, 1993; Schimke, 1988). Therefore, it is important to determine the mechanisms by which cells can become resistant to them. Accordingly, we have used expression cloning to isolate cDNAs containing plasmids that cause MCF-7 breast cancer cells to become resistant to immunotoxins (Brinkmann et al., 1995b). These plasmids also render the cells resistant to native Pseudomonas exotoxin and diphtheria toxin; thus the resistance is due to the action of the toxin moiety of immunotoxins and not due to reduced expression of the antigen to which the immunotoxin is directed. One of these cDNAs was particularly interesting because its phenotype could not be attributed to known mechanisms of PE and DT resistance (Carroll & Collier, 1987; Fendrick et al., 1992; Kido et al., 1991). In fact, when cells transfected with this plasmid were exposed to toxin, modification of EF2 and inhibition of protein synthesis were comparable to toxin sensitive controls, yet the cells did not die. It appeared as if this cDNA was affecting the sensitivity of cells to toxin after the primary action of the toxin, inhibition of protein synthesis, had occurred. The observation that PE and DT can induce apoptosis made us suspect that this cDNA could be interfering with apoptosis.

Here we analyze the mechanism by which one cDNA plasmid mediates toxin resistance to MCF-7 cells without interfering with ADP-ribosylation of EF2 and subsequent inhibition of cellular protein synthesis. This plasmid has already been shown to contain an antisense cDNA fragment of a human *CAS* gene (Brinkmann et al., 1995a), but the mechanism by which CAS antisense mediates toxin resistance was not investigated. *CAS* is the human homologue to the yeast *CSE1* gene, which plays a role in chromosome segregation as well as in B-type cyclin degradation (Xiao et al., 1993; Irniger et al., 1995). We demonstrate that MCF-7 cells containing a plasmid that expresses CAS (CSE1 homologue) antisense cDNA are less susceptible to toxin mediated apoptosis as well as to apoptosis induced by TNFα and -*β*.

* Author to whom all correspondence should be addressed. Phone (301) 402-2708; fax (301) 402-1344.

[⊗] Abstract published in *Advance ACS Abstracts,* May 1, 1996.

[1] Abbreviations: PE, Pseudomonas exotoxin; DT, diphtheria toxin; TNF, tumor necrosis factor.

S0006-2960(95)02829-7

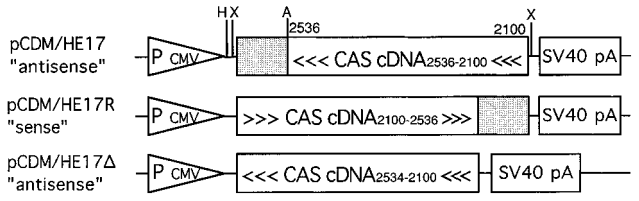

FIGURE 1: Plasmid constructions: pCDM/HE17 was obtained from a pCDM8/HeLa cDNA expression library by immunotoxin selection cloning as a plasmid that renders MCF-7 cells resistant to immunotoxins (see Experimental Procedures). It contains a 700 bp insert composed of 436 bp antisense cDNA to the human *CAS* gene, a homologue of the yeast *CSE1* gene, and 264 bp unrelated sequence (shadowed) in an antisense direction behind the CMV promoter followed by the SV40 poly(A) signal. X, A, and H indicate *Xba*I, *Asp*700, and *Hin*dIII sites. pCDM/HE17R contains the cDNA (*Xba*I fragment) of pCDM/HE17 in reverse orientation, and pCDM8/HE17Δ has the 260 bp *Asp*700−*Hin*dIII non-CSE1 fragment deleted.

EXPERIMENTAL PROCEDURES

Plasmid Constructions. A cDNA library in pCDM8 that contains HeLa cDNA expressed from a CMV promoter and followed by a SV40 poly(A) sequence was obtained from Clontech. pCDM/HE17 (p17 in ref. Brinkmann et al., 1995b) was isolated from that library by immunotoxin selection and contains a 700 bp insert composed of 436 bp antisense cDNA to the human homologue of the yeast *CSE1* gene and 264 bp unrelated sequence. We used standard cloning techniques (Maniatis et al., 1989) to make pCDM8/HE17R which contains the 700 bp cDNA in an inverse orientation, and pCDM8/HE17Δ that has the non-CSE1 sequence deleted (as a 260 bp *Asp*700−*Hin*dIII fragment) (Figure 1). pCDM8/C2−C5 were randomly chosen from our library and contain unknown inserts. pCDM8 derived plasmids were propagated in *Escherichia coli* MC1061/P3 with ampicillin/tetracycline selection. Plasmid DNA for transfections was purified by Qiagen plasmid DNA preparation kits.

Expression Cloning and Immunotoxin Selection of cDNA Plasmids. Human cDNAs that confer resistance to the immunotoxin B3(Fv)-PE38KDEL were isolated by expression cloning and immunotoxin selection (Brinkmann et al., 1995b). B3(Fv)-PE38KDEL is a fusion protein composed of a truncated form of PE and the Fv region of MAb B3 that binds to a carcinoma related carbohydrate on many carcinomas and cancer cell lines, e.g., MCF-7 breast carcinoma cells (Brinkmann et al., 1991), and subsequently kills such cells. Briefly, MCF-7 cells expressing SV40 T antigen (MCF-7/T; Brinkmann et al., 1995b) were transfected with a HeLa cDNA expression library and treated 2 days later with high doses of B3(Fv)-PE38KDEL (Brinkmann et al., 1991). Dead cells were removed by washing with PBS, remaining cells harvested, and plasmids recovered (Hirt, 1967; T-antigen allows episomal replication), amplified in *E. coli*, and retransfected into MCF-7/T for further rounds of immunotoxin selection and plasmid reisolation. Plasmids that cause cells to survive after immunotoxin exposure are selectively enriched by this procedure, resulting in siblings with the same or similar selected cDNAs in the plasmid pool. Such plasmids were identified by hybridization analysis, and several plasmids were found that were present more than once in a pool of 96 single plasmid clones randomly isolated after three rounds of immunotoxin selection. These were chosen as resistance plasmid candidates and stably transfected into MCF-7 cells to analyze individually their effects on toxin sensitivity (Brinkmann et al., 1995b). MCF-7/17 contains such a plasmid (pCDM/HE17; p17 in Brinkmann et al., 1995b) and was obtained by cotransfection with pMC1neo/poly(A) and subsequent G418 selection.

Recombinant Cell Lines. Cell lines were produced by electroporation of 5×10^6 MCF-7 cells with 3 μg of pMC1neo/poly(A) and 15 μg of expression plasmid, using a BioRad gene pulser at 400 V, 960 FD, in 0.4 cm cuvettes as described (Brinkmann et al., 1995b). MCF-7/T is a pool of MCF-7 cells that express SV40 large T antigen, thus allowing episomal replication of plasmids with an SV40 origin, e.g., pCDM8. MCF-7/T were made by cotransfection of pCMV-TAg (Ogryzko et al., 1994) and pMC1neo/poly(A) and selection of a transfected cell pool with 0.8 mg/mL G418 (Brinkmann et al., 1995b; see also Figure 1). Similarly, MCF-7/17, MCF-7/17R and MCF-7/17Δ are cell pools produced by cotransfection of pMC1neo/poly(A) and 15 μg of either pCDM/HE17, pCDM/HE17R, or pCDM/HE17Δ, respectively. MCF-7/C and MCF-7/C2-C5 are cotransfectants of pMC1neo/poly(A) with pCDM8 (vector alone, MCF-7/C) or pCDM/C2-C5, control plasmids randomly chosen from the library without any selection. MCF-7/N contains only pMC1neo/poly(A). All these cell lines were selected and propagated with 0.8 mg/mL G418 and were not exposed to toxins or TNF until their response to these agents was tested.

Cytotoxicity Assays. PE and PE-derived immunotoxins were produced by us; DT was obtained from Sigma, recombinant human TNF α from Promega, and TNF β from Boehringer Mannheim. Sensitivity toward these agents was assayed by MTS assays (Promega; Cory et al., 1991) which detect dehydrogenases present in living cells (A_{490nm} is proportional to number of live cells), or by assaying incorporation of [^{3}H]leucine into cellular proteins (protein synthesis inhibition assay; Brinkmann et al., 1991). If not noted otherwise, 3×10^3 cells/well in 200 μL of medium were plated and grown overnight, incubated with toxin or TNF for 20 h or as indicated, and then assayed.

ADP-Ribosylation of Cell Extracts. Extracts for assaying ADP-ribosylation of EF2 by PE were prepared by suspending PBS washed cell pellets in lysis buffer (10 mM Tris, 10 mM KCl, 1.5 mM Mg acetate, 6 mM mercaptoethanol, pH 7.5) for 30 min at 20 °C followed by homogenization. Then 0.2 volume of 1.25 M sucrose was added, the suspension centrifuged (30 min, 4 °C, 100000*g*), and the supernatant dialyzed against lysis buffer containing 0.25 M sucrose. Equal (protein) amounts of extracts were incubated in 250 μL (final volume, adjusted with 50 mM Tris, 1 mM EDTA) of assay buffer containing 40 mM DTT, with 1.5 μL of [^{14}C]-NAD (Amersham, 287 mCi/μmol, 250 μCi/mL) and 100 ng/mL PE (final concn) for 30 min at 37 °C. Protein-associated radioactivity was precipitated with 12% TCA, washed with 6% TCA, solubilized with 0.1 M NaOH, and neutralized with HCl and radioactivity counted in scintillation liquid.

TNF Binding. Binding assays were performed essentially as previously described (Webber et al., 1995). A total of 3×10^5 cells per dish in 24-well plates were chilled and blocked with RPMI/5% BSA/50 mM BES, pH 7, for 1 h at 4 °C and washed twice with binding buffer (RPMI/1% BSA/50 mM BES, pH 7). ^{125}I-Labeled TNFα and cold TNF in binding buffer were then added and incubated for 2 h. For competition experiments, 80 pM (final concentration) of ^{125}I-

Table 1: The Immediate Actions of PE and TNF Are Not Affected in CAS Antisense Containing Cells[a]

		MCF-7, MCF-7/N (control)	MCF-7/17 (CAS antisense)
PE	ADP-ribosylation act. (cpm)	1328 ± 250	1329 ± 200
	protein synthesis inhibn (IC_{50}) (ng/mL)	0.5−1	0.5−1
	cell death (LC_{50}) (ng/mL)	1−2	15−20
TNF	receptors/cell	17000 ± 4000	17000 ± 5000
	affinity (pM)	102 ± 8	104 ± 17
	cell death (LC_{50}) TNF α (ng/mL)	0.15	1−3
	cell death (LC_{50}) TNF β (ng/mL)	1−3	20−30

[a] ADP-ribosylation of EF2 in cell extract was assayed as described in the Experimental Procedures. Inhibition of protein synthesis was measured by incorporation of [^{3}H]leucine 15 h after toxin exposure, and cell death was assessed by MTS assays (see Experimental Procedures and Figures 2 and 5). IC_{50} is the toxin concentration that reduces protein synthesis by 50% compared to untreated cells, and LC_{50} is the concentration that kills 50% of the cells (MTS assays). The number of TNF receptors per cell and affinity were determined by ^{125}I-TNFα (Amersham) competition and displacement assays using the program Ligand for data processing (Munson & Rodbard, 1983). The mean of those experiments ±SE is shown; within this error range, displacement assays gave slightly lower apparent receptor number and higher affinity than the mean and competition assays showed slightly lower affinity and higher receptor numbers.

TNF, 12 nCi, 16 fmol/well (Amersham), was mixed with varying concentrations of unlabeled TNF. For Scatchard experiments, serial dilutions, 18−70 pM, of labeled TNF were used. Labeled TNF was then removed, and the cells were washed twice with binding buffer. Cell bound radioactivity was recovered in 0.5% SDS in TE and determined in a Beckman 5500B counter. Receptor numbers and affinity was calculated using the Ligand program (Munson & Rodbard, 1983).

DNA Degradation. Release of DNA fragments into cell culture supernatant was analyzed by labeling cells for 20 h with [^{3}H]thymidine (Amersham), changing medium, and exposing cells to toxin as described (Kochi & Collier, 1993). DNA release was assayed by measuring radioactivity in the medium, in floating cells and in the soluble fraction of attached cells and compared with the total radioactivity (Kochi & Collier, 1993). When analyzing internucleosomal DNA degradation, we found that DNA of cells still attached to culture dishes was in most cases a smear (50 000−200 bp) and the ladder fragments were difficult to visualize. However, DNA obtained from cell culture fluid containing floating cells by phenol/chloroform extraction and ethanol precipitation clearly showed a ladder.

Western Analysis with Anti-CAS Antibodies. Polyclonal antibodies that specifically recognize human CAS were obtained by immunizing rabbits with recombinantly produced His-tagged CAS protein fragments (either amino acids 1−284 or 327−669 of the sequence in ref Brinkmann et al., 1995a) that were affinity-purified on Ni affinity columns (Qiagen). Details of the protein fragment preparation, immunization, and the antibodies obtained are described by Scherf et al. (1996). Comparison of prebleeds and sera from the immunized rabbits shows a ∼100 kDa cellular protein that is specifically recognized using sera from immunized rabbits, independent of whether the amino terminal or central CAS protein fragment was used as immunogen. The molecular mass of CAS protein deduced from its cDNA reading frame is 110 kDa. Thus a ∼100 kDa band is the expected size on reducing SDS−PAGE. For quantification of CAS protein by Western analysis, total cell extracts were first carefully adjusted to contain equal concentrations of protein using Coomassie blue stained SDS−PAGE, and then equal amounts were transferred to nitrocellulose. CAS protein on these blots was detected with anti-CAS serum, biotin−anti-rabbit IgG and avidin−HRP, and Amersham ECL reagents following the Amersham ECL protocol. The amount of CAS on the filters was quantified using an Ambis (Quantprobe) image analyzer.

Table 2 [a]

treatment	LC_{50} MCF-7/N	LC_{50} MCF-7/17	*x*-fold resistance
PE	1−2 ng/mL	15−20 ng/mL	∼10
DT	0.006 ng/mL	0.07 ng/mL	∼10
TNFα	0.15 ng/mL	1−3 ng/mL	∼10
TNFβ	1−3 ng/mL	20−30 ng/mL	∼10
VP16	30 μg/mL	30 μg/mL	1
staurosporine	1−2 ng/mL	1−2 ng/mL	1
cycloheximide	∼300 ng/mL	∼250 ng/mL	1

[a] MCF-7/17 (CAS antisense) cells are resistant to ADP ribosylating toxins and TNF but not generally resistant to apoptosis-inducing agents. Cytotoxicity was determined by MTS assays (PE and DT) or protein synthesis inhibtion assays (see Experimental Procedures). Some previously published data (Brinkmann et al., 1995b) are included. LC_{50} is the concentration that kills 50% of the cells as assessed by MTS assays (PE, DT, cycloheximide, TNF, staurosporine) or [^{3}H]leucine incorporation (VP16).

RESULTS

CAS Antisense Renders MCF-7 Cells Resistant to Pseudomonas and Diphtheria Toxin. pCDM/HE17 is a plasmid containing a 700 bp HeLa cDNA that was isolated by a combination of expression cloning and immunotoxin selection from a HeLa cDNA library transfected into MCF-7 breast cancer cells (Figure 1, Table 1). It renders MCF-7 cells ∼10-fold less sensitive toward a PE-derived immunotoxin as well as to native PE and DT (Brinkmann et al., 1995b). Both of these toxins cause cell death by ADP-ribosylating and inactivating EF2 and thereby arresting protein synthesis. Cells containing this plasmid are not resistant to ricin or cycloheximide which inhibit protein synthesis by other mechanisms, nor are they protected against cell death induced by various chemotherapeutic agents, e.g., VP16, or by staurosporine (Table 2). We have previously shown that pCDM/HE17 contains a 436 bp cDNA fragment that is part of a human homologue of the yeast *CSE1* chromosome segregation gene (Brinkmann et al., 1995a). This cDNA fragment which contains nucleotides 2100−2536 of the full length clone is present in an "inverse" orientation so that transcription from the CMV promoter of pCDM/HE17 generates antisense RNA (Figure 1). pCDM8/HE17 also contains 264 bp of an unrelated sequence fused to CAS antisense. This appears to be a library-ligation artifact since this sequence is not related to the sequence of the full length

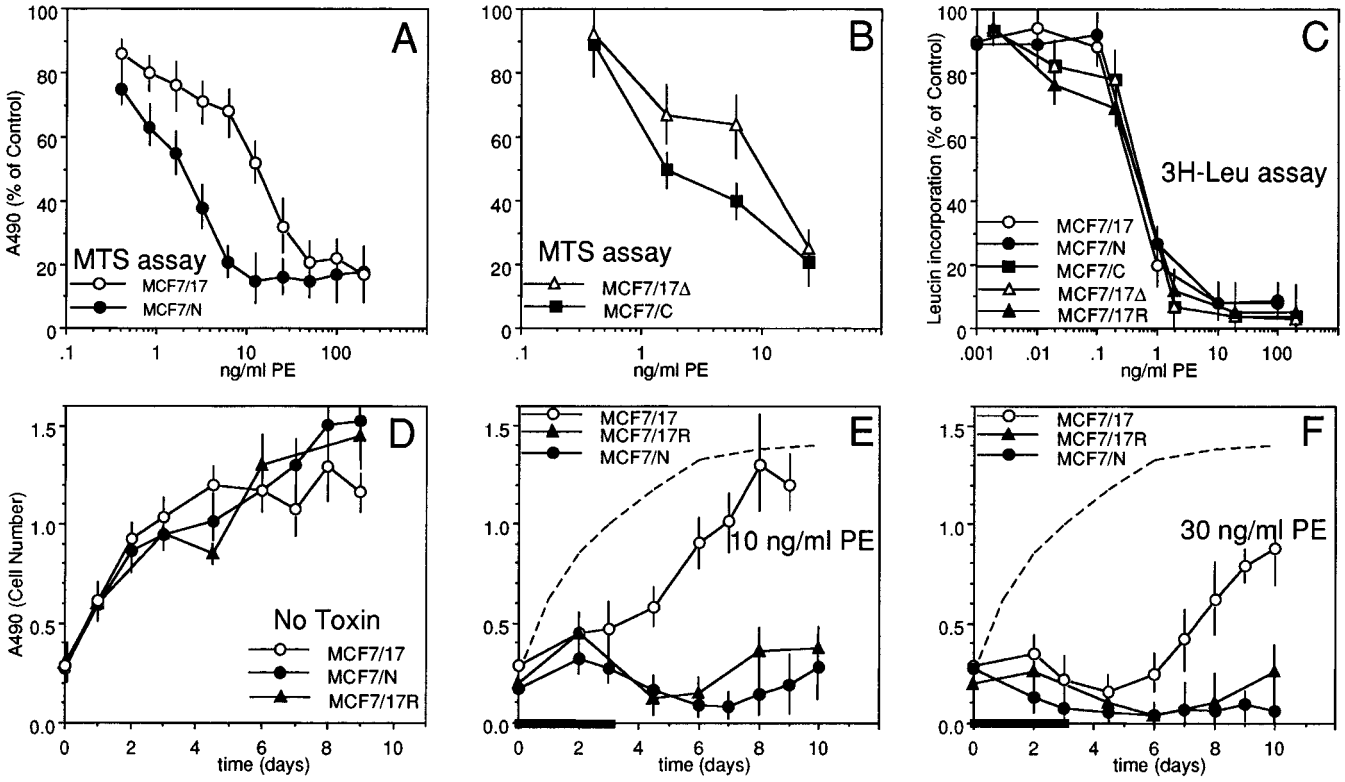

FIGURE 2: CAS antisense reduces the sensitivity of MCF-7 cells to PE: (A, B) The number of viable cells surviving toxin exposure was determined by MTS assay 3 days after toxin addition. The data in (A) have been previously shown (Brinkmann et al., 1995b) and were included to demonstrate the phenotype of toxin resistance with affect on protein synthesis inhibition. (C) Inhibition of protein synthesis was assayed by measuring the incorporation of [^{3}H]leucine 20 h after toxin addition. (D and E) Time course experiments showing antisense tranfectants undergo growth arrest upon toxin treatment but recover after removal of toxin. Cell viability was determined by MTS assays in 96-well plates. A_{490} is proportional to the number of cells in each sample. MCF-7/17 (CAS antisense) cells showed growth about equal with the control cells, but a longer lag phase before resuming growth after trypsinization (not shown in this figure) and often slightly reduced incorporation of [^{3}H]leucine in protein synthesis inhibition experiments (although these differences were small and inconsistent). A typical growth curve (mean of three experiments) of cells without toxin is indicated by a broken line. The duration of toxin exposure is indicated by a bar. The symbols are the same for all experiments. MCF-7/17 (○) and MCF-7/17Δ (△) contain CAS antisense. Controls are inhibtion MCF-7/N cells (●), which contain only the pMC1neo/poly(A) plasmid but not any library plasmid, or MCF-7/C cells (■), which contain pMC1neo/poly(A) and the pCDM8 library vector without insert, and MCF-7/17R cells (▲), which contain *CAS* cDNA in the sense direction (see Figure 1B).

CSE1 homologue isolated from human placenta cDNA and also not linked to the gene in HeLa cells, as shown by hybridization of Southern blots of HeLa and placenta DNA cleaved with various enzymes (data not shown). Furthermore, this unrelated sequence does not have an open reading frame and therefore is noncoding, and its expression cannot be detected by Northern blot analysis in any human tissue (data not shown). Accordingly, we reasoned that the resistance mediated by pCDM/HE17 is likely due to antisense RNA to human *CAS*.

To determine if toxin resistance mediated by pCDM/HE17 is caused by antisense RNA production and subsequent interference with expression of the human *CSE1*-homologue gene *CAS*, we constructed a plasmid in which the insert of pCDM/HE17 was inverted (pCDM/HE17R) so that a sense RNA fragment would be made (see Figure 1). To rule out a possible effect of the additional non-CSE1-like sequence, we also deleted the non-CSE1-like sequence (pCDM/HE17Δ). We then cotransfected these plasmids with pMC1neo/poly(A) into MCF-7 cells and isolated pools of stably transfected cells containing pMC1neo/poly(A) and each of these plasmids. All transfectants, including the original MCF-7/17, were not isolated as single clones from transfections but as pooled transfectants (>10 colonies) to compensate for possible clonal variability in the cells containing the transfected plasmids (Brinkmann et al., 1995b). Additional controls were MCF-7 cells cotransfected with pMC1neo/poly(A) and the library vector pCDM8 without insert (MCF-7/C), MCF-7 cells with randomly chosen plasmids from the unselected pCDM8/HeLa library (MCF-7/C2−C5), and MCF-7 cells with pMC1neo/poly(A) alone (MCF-7/N). Cells were propagated under G418 selection and not exposed to toxin before their response to toxins was analyzed.

Figure 2 shows experiments in which the sensitivity of MCF-7 cells and CAS antisense transfected MCF-7 cells toward Pseudomonas exotoxin was analyzed using the MTS cell proliferation assay (see Experimental Procedures). PE was chosen to represent ADP-ribosylating toxins (immunotoxins, DT, PE) in this study because the relative sensitivities or resistances of the cell lines to PE, DT, and immunotoxins were comparable; i.e., cells resistant to PE also were resistant to immunotoxin as well as to DT (Brinkmann et al., 1995b, and data not shown). Figure 2A shows an experiment in which cells were exposed to various concentrations of PE for 3 days and the number of cells present on day 3 measured by the MTS assays. It is evident that MCF-7/17 (CAS antisense) cells are ~10-fold more resistant to PE than the control cell line (MCF-7/N) and MCF/C (data not shown) with an IC_{50} of ~15 ng/mL compared to 1−2 ng/mL in the control. To eliminate the possibility that the additional non-CAS sequence influences toxin sensitivity, we tested MCF-

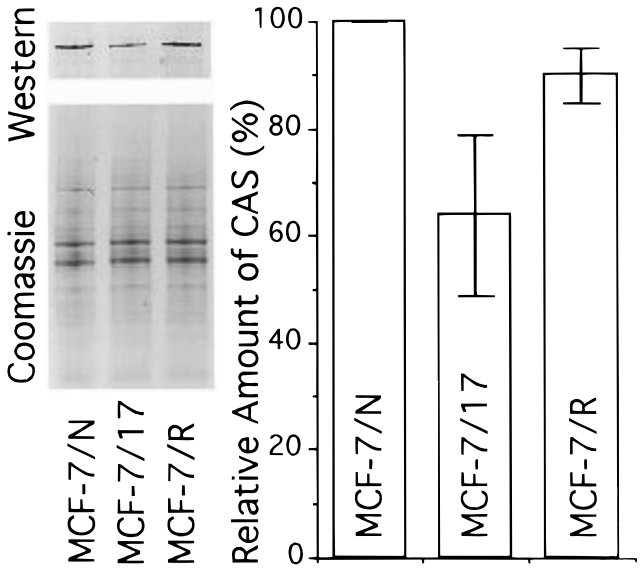

FIGURE 3: Detection of CAS protein by Western blot. Equal amounts of total cell extracts of MCF-7/17, MCF-7/N, and MCF-7/R cells were separated on a 4−15% reducing SDS−PAGE and stained with Coomassie blue to demonstrate equal loading (lower panel) or analyzed by Western blot (upper panel) and image quantification of the Western blot (see Experimental Procedures). Polyclonal anti-CAS antibodies raised in rabbits were used to detect CAS protein. CAS appears as ~100 kDa protein, and CAS levels are lower in MCF-7/17 CAS antisense cells than in the control cells. The bars indicate variations in CAS quantification from different Western blots.

7/17Δ cells containing CAS antisense without the additional sequence. Figure 2B shows that these cells were also more resistant to PE, although the effect (resistance) was not as pronounced as with MCF-7/17. The reason for this difference is unknown. These experiments show that the presence of CAS antisense reduces the sensitivity of MCF-7 cells toward PE.

Reduced CAS Protein Levels in CAS Antisense Containing Cells. One explanation for the effects of the CAS antisense plasmid, i.e., protection against toxin mediated cell death, is interference with expression of the corresponding cellular gene. It is known that expression of antisense cDNA fragments can reduce the levels of expression of cellular genes (Gudkov et al., 1993, 1994). To evaluate this possibility, we examined the levels of CAS protein in CAS antisense containing MCF-7/17 and control cells by Western blots using CAS specific polyclonal antibodies (see the Experimental Procedures, Figure 3). We found equal amounts of the 100 kDa CAS protein in the control cells MCF-7/N and MCF-7/R. However, in the CAS antisense containing MCF-7/17 cells, the CAS protein levels were reproducibly found to be reduced to 64 ± 15%, compared to 100% in the controls (Figure 3). We conclude that the intracellular level of CAS protein is reduced in CAS antisense containing cells. This reduction is the probable reason for the observed toxin resistance of these cells. We could not obtain a complete (or greater) reduction in CAS protein levels using this antisense approach, because further experiments showed that high-level CAS antisense expression renders cells nonviable (our unpublished results, see Discussion).

CAS Antisense Reduces the Susceptibility of Cells to Apoptosis Induced by ADP-Ribosylating Toxins. How does CAS antisense render cells resistant to ADP-ribosylating toxins? Several possibilities by which cells can become resistant are known, which include defects in toxin binding, internalization, processing, and alterations in EF2, the intracellular toxin target (Fendrick et al., 1992; Kido et al., 1991; Chaudhary et al., 1990; Laurie & Robbins, 1991). When we examined the ability of PE to inhibit the incorporation of [^{3}H]leucine into MCF-7 cells (the standard assay to quantitate PE mediated toxicity), we found that none of these mechanisms can be responsible for the resistance. As shown in Figure 2C, there was no difference in the incorporation of leucine between CAS antisense and the control cell lines, even though the MTS assay showed that the toxin sensitivity was very different between the lines. In these experiments, we included an additional control which is expressed in the sense direction (MCF-7/R). Furthermore, the direct measurement of the ability of PE to ADP-ribosylate EF2 in extracts of CAS antisense MCF-7/17 cells and in control cells shows that the sensitivity of EF2 in the toxin resistant transfectant was indistinguishable from controls (Table 1). Thus, CAS antisense does not make cellular EF2 resistant to PE; EF2 becomes modified as effectively as in control cells.

To gain more information about the status of the cells that are resistant to PE, time course experiments were carried out at two concentrations of PE (10 and 30 ng/mL). These concentrations were chosen because they had maximal inhibitory effect on the growth of control cells (Figure 2A) and maximally inhibited protein synthesis (Figure 2C). In addition, cells containing sense plasmids as well as other controls were included. Figure 2D−F shows the time course of treatment with PE. MCF-7/17 (CAS antisense) cells treated with 10 ng/mL PE for 3 days (Figure 2E) showed little or no growth compared to untreated cells (compare to Figure 2D). However, when the PE was removed, the cells began to grow and by day 8 reached almost the same cell number as control cells not treated with PE. Figure 2F shows that MCF-7/17 cells treated with 30 ng/mL of PE for 3 days showed no growth, but after removal of PE, recovered and resumed growth. In contrast, MCF-7/N cells which contained only the pMC1neo/poly(A) plasmid and MCF-7/17R cells which contained in addition the pCDM/HE17R (CAS sense) showed no growth in the presence of 10 or 30 ng/mL PE and did not recover after toxin removal (Figures 2E and 2F). This phenotype was also observed with the other control plasmids MCF-7/C and MCF-7/C2-C5 (Experimental Procedures and data not shown). From these experiments we draw two conclusions. One is that, in contrast to control cells which die as a consequence of toxin treatment, cells containing the CAS antisense plasmid remain alive and begin to proliferate when the toxin is removed. The other is that the effect of the antisense plasmid is specific because cells containing the sense plasmid (MCF-7/17R) as well as other control plasmids did not recover after toxin removal.

Figure 4 shows the morphologic appearance of MCF-7/N cells and MCF-7/17 cells (containing the antisense plasmid) without toxin treatment and treated with 10 ng/mL PE for 2 days. The untreated cells grow as flattened cells in islands characteristic of MCF-7 cells. There was no obvious morphological difference between the MCF-7/N cells and MCF-7/17 cells. However, the appearance of the cells treated with 10 ng/mL PE for 2 days was very different. No MCF-7/N cells could be detected attached to the dish; all the cells were floating and refractile, and many were

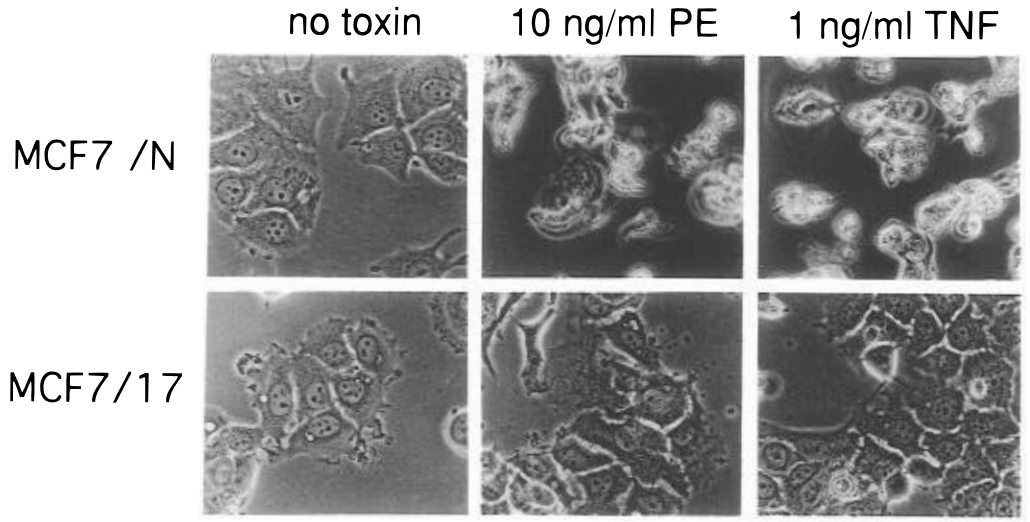

FIGURE 4: Morphology of cells after exposure to PE and TNF. Phase contrast microscopy of a random field at 250× magnification after incubation with PE (10 ng/mL) or TNFα (1 ng/mL) for 3 days. Control cells (MCF-7/N) become rounded and refractile, and detach. The CAS antisense transfected MCF-7/17 cells remain attached.

disintegrating. In contrast, most of the MCF-7/17 cells remained flattened and attached to the dish and resembled cells that had not been treated with PE even though their ability to incorporate [^{3}H]leucine into protein was arrested to background levels (see Figure 2C at 10 ng/mL PE). Presumably these, are the cells that resume growth when toxin is removed (Figure 2E,F).

Since some cells undergo apoptosis upon exposure to ADP-ribosylating toxins (Chang et al., 1989; Kochi & Collier, 1993; Morimoto & Bonavida, 1992), one possible explanation for the fact that MCF-7 cells containing the CAS antisense plasmid become less sensitive to toxins without changing their susceptibility to toxin mediated protein synthesis inhibition is that the CAS antisense affects the susceptibility of cells to undergo apoptosis. One hallmark of apoptosis is DNA degradation and the formation of a DNA ladder due to internucleosomal cleavage of chromosomal DNA (Compton, 1992). Therefore, DNA was obtained from cells treated with PE, and the DNA in floating cells, in the cell culture medium, and in the soluble cell fraction of attached cells was analyzed as described by Kochi and Collier (1993). As shown in Figure 5A, with increasing concentrations of PE, there is a concomitant increase in DNA released into the medium and into the soluble fraction of cells. Since intact genomic DNA is usually not present in the culture medium, and the soluble cell fraction of healthy cells, this indicates extensive DNA degradation upon toxin treatment of control cells. In contrast, in MCF-7/17 CAS antisense cells, the production of "soluble" DNA was greatly diminished. To examine the nature of the released DNA, DNA was isolated from the medium which contained floating cells where most of the "soluble" DNA was located and subjected to electrophoresis.

Figure 5B shows that PE treated MCF-7/N cells display a typical nucleosomal ladder, whereas, the MCF-7/17 cells show mostly high molecular weight fragments and a very faint DNA ladder that could not be photographically reproduced. Thus, DNA degradation and nucleosomal ladder formation are diminished in MCF-7/17 cells containing CAS antisense. These results show that CAS antisense interferes with a pathway downstream of protein synthesis but upstream of the "execution phase" of apoptosis because it is common

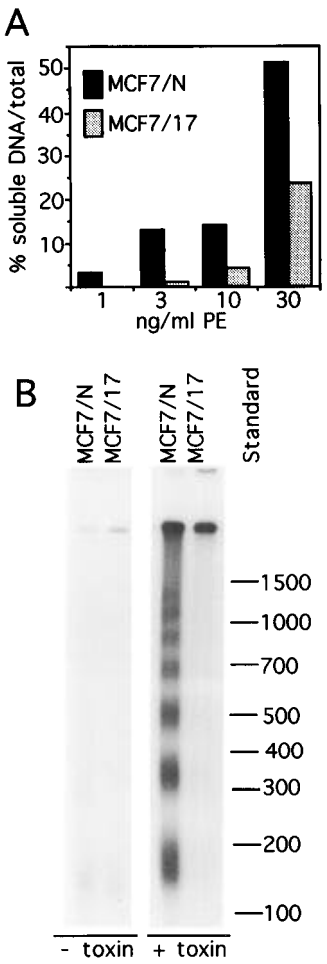

FIGURE 5: CAS antisense renders MCF-7 cells less susceptible to apoptosis. (A) Toxin treatment induces degradation of chromosomal DNA. 10^6 cells were labeled with [^{3}H]thymidine (1 mCi/mL) overnight and treated with various amounts of PE for 15 h, and the amount of [^{3}H]thymidine in chromosomal DNA, the cellular soluble fraction in floating cells and in the medium were collected by scintillation counting as described (Kochi & Collier, 1993). (B) Toxin treatment generates a nucleosome ladder. ^{3}H-Labeled DNA (floating cells and medium) was isolated (see A) by phenol extraction and ethanol precipitation and electrophoresed on a 1.5% agarose gel. The autoradiograph shows a DNA ladder in control cells but very little in MCF-7/17 CAS antisense cells.

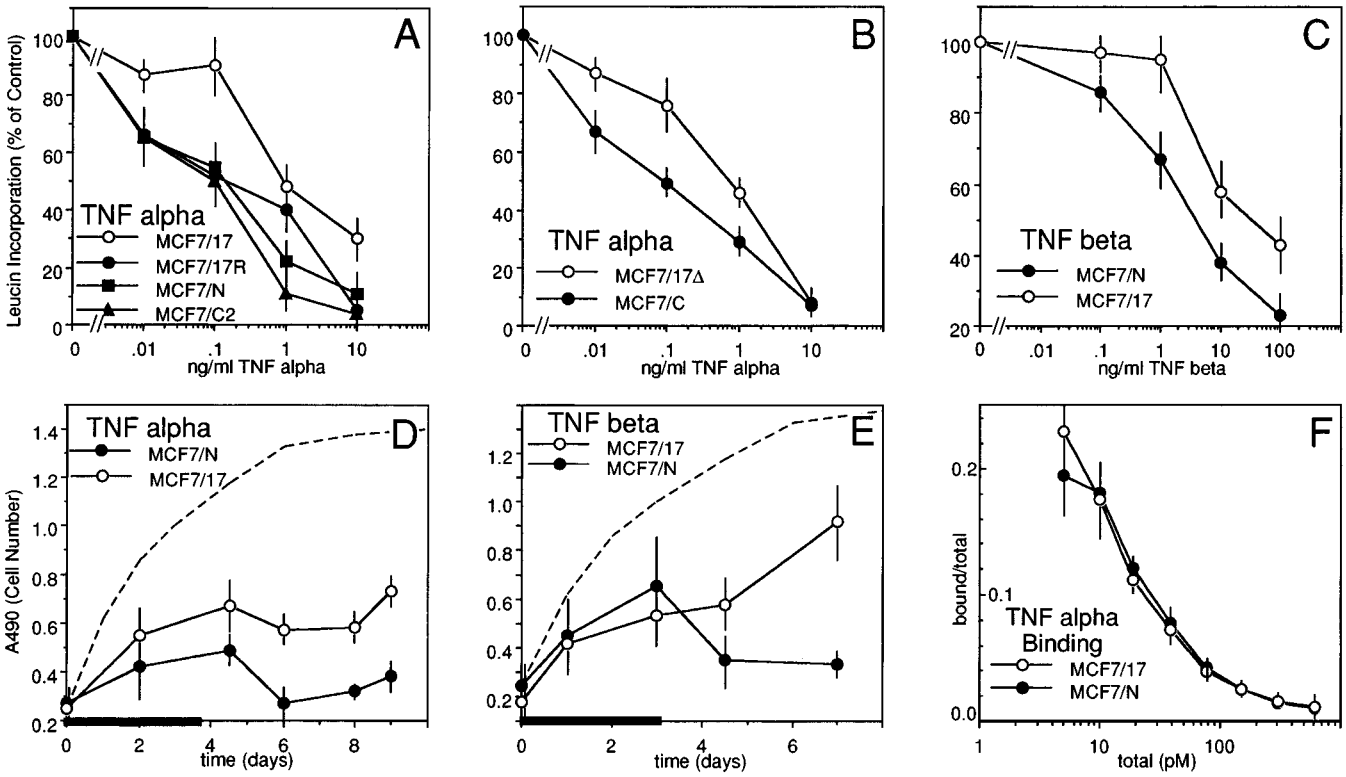

FIGURE 6: CAS antisense reduces the sensitivity of MCF-7 cells to TNF. (A, B, C) [^{3}H]leucine incorporation assays were used to quantitate killing by TNF. The assays were performed 3 days after TNFα (A and B) and TNFβ (C). (D, E) Time course experiments. Cell viability was determined by MTS assays in 96-well plates as described in Figure 2. An average growth curve (mean of three experiments) of cells without toxin is indicated by a broken line, and the duration of TNF exposure is indicated by a bar. MCF-7/17 and MCF-7/17Δ contain CAS antisense. Controls are MCF-7/17R that contains the sense plasmid, and MCF-7/C that contains the pCDM8 library vector without insert and MCF-7/C2 that contains a randomly chosen library plasmid with an unknown insert (see Figure 1). (F) TNFα receptor numbers and affinity were determined using ^{125}I-TNF (Experimental Procedures and Table 1).

to ADP-ribosylating toxins and TNF (see below) but not to cycloheximide.

The CAS Antisense Plasmid Reduces the Susceptibility of MCF-7 Cells to TNF. It is well established that apoptosis can be induced in certain cell lines by TNF α and TNF β. Although PE and DT on one hand and TNF on the other hand produce cell death by different mechanisms [the toxins cause inhibition of protein synthesis by modification of EF2, while TNF acts by receptor binding and signal transduction (Smith et al., 1994)], it has been suggested that DT and TNF mediated cytotoxicity might share a pathway that leads to cell death (Morimoto & Bonavida, 1992). Therefore, it might be possible that cells that are resistant to PE might also be more resistant to TNF. Because MCF-7 cells express the TNF receptor (Table 1) and are sensitive to TNF mediated apoptosis, we could test this possibility. Figure 6 shows the effects of CAS antisense plasmids on TNF induced apoptosis in MCF-7 cells. The IC_{50}s of TNFα on untransfected MCF-7 cells and cells containing control plasmids are approximately 0.1−0.2 ng/mL (Figure 6A,B). The IC_{50} of TNFβ was 2−3 ng/mL for the control (Figure 6C). In contrast, the CAS antisense plasmid bearing MCF-7/17 cells is ∼5−10-fold less sensitive both to TNFα (IC_{50} ∼1 ng/mL) and to TNFβ (IC_{50} ∼20 ng/mL). This difference in TNF sensitivity between control cells and CAS antisense cells can also be easily seen by the morphological appearance of TNF treated cells (Figure 4). Control cells treated with 1 ng/mL TNF were detached from the dishes and were disintegrating, while CAS antisense cells stayed attached and resembled untreated cells. This phenotype is very similar to the effects seen with PE treated cells. Also similar to the phenotype of CAS antisense transfected MCF-7 cells that are treated with PE, TNF treatment of these cells leads to growth inhibition, which can be overcome after TNF removal (see Figure 6D for TNFα and Figure 6E for TNFβ, compare to Figure 2D−F). The control cells do not survive TNF treatment and do not recover after TNF removal. The altered sensitivity of cells to TNF is not due to interference with the immediate action of TNF, which is binding to its receptor. Figure 6F and Table 1 show that the TNF receptor numbers and affinity remained unchanged in CAS antisense containing MCF-7/17 cells. We conclude that the CAS antisense plasmid inhibits some event in the TNF mediated apoptosis pathway downstream of TNF binding, and it is likely to be in a pathway that is common for TNF and for ADP-ribosylating toxins.

DISCUSSION

We have recently isolated a human cDNA containing plasmid that renders MCF-7 breast carcinoma cells resistant to the cytotoxic effects of Pseudomonas toxin and diphtheria toxin as well as to TNFα and TNFβ. This cDNA is an antisense fragment of *CAS*, a cell proliferation/division related human homologue of the yeast *CSE1* gene (Brinkmann et al., 1995a,b; Xiao et al., 1993). It mediates resistance because expression of the cDNA fragment generates antisense RNA to *CAS*, which renders cells less sensitive to toxin and TNF mediated apoptosis, whereas expression of a sense RNA does not. This suggests that *CAS* might have two functions: one in cell proliferation and division as previously suggested (Brinkmann et al., 1995a), and

another, as shown here, in apoptosis induced by ADP-ribosylating toxins and TNF.

The conclusion that CAS antisense cDNA reduces the sensitivity of cells to toxins and TNF is based on several observations: (i) the CAS antisense plasmid was obtained by expression cloning and immunotoxin selection, a method that selectively enriches plasmids that confer resistance against toxin and immunotoxin mediated cell death (Brinkmann et al., 1995b); (ii) resistance to toxins as well as to TNF is specific to CAS antisense; neither control plasmids nor the corresponding sense fragment causes resistance; (iii) the resistance is unrelated to the primary action of PE, DT, and TNF, and neither the immediate toxin action (on EF2) nor TNF binding is affected by CAS antisense; furthermore, instead of dying like normal MCF-7 cells after exposure to toxin or TNF, the transfectants can recover after removal of toxin or TNF; (iv) the formation of a DNA ladder caused by internucleosomal DNA cleavage is observed in MCF-7 cells exposed to toxin or TNF, and it is much less pronounced in CAS antisense containing cells under identical conditions.

How does CAS antisense inhibit apoptosis? It is well established that expression of antisense cDNA fragments can interfere with expression of corresponding cellular genes. This principle has been used for selection cloning of cDNA fragments that mediate drug resistance (Gudkov et al., 1993, 1994). Therefore, we measured the level of CAS protein in the cell population containing CAS antisense and found it to be reduced by ~40%. This fall in CAS levels appears to be sufficient to inhibit toxin and TNF mediated apoptosis although not completely (Figures 2 and 5). One reason for only a 40% fall in CAS may be that we analyzed the effect of CAS antisense on a pool of transfected cells instead of single clones. Probably cells with lower CAS levels did not grow. In other experiments we found that transfection of MCF7 cells by full length CAS antisense cDNA causes cell death. These results were not surprising since one would expect growth inhibition upon effective antisense inhibition of an essential cell proliferation gene (Brinkmann et al., 1995a). This is also consistent with the fact that in yeast the homologue *CSE1* is essential and homozygous *CSE1* mutants are not viable. One could expect various possibilities for cells to escape from CAS antisense mediated inhibition, particularly when cells are grown for many passages due to the necessity of expanding cell populations from single clones. The analysis of single clones has the risk of selecting for different responses in different clones. By analyzing transfected pools instead of clones, one can obtain a mean of the different effects of different levels of antisense expression on the whole cell population. We believe that our approach of pooling clones is a resonable compromise between obtaining cells with sufficient expression of CAS antisense and avoiding selection of various compensatory events.

The results that we obtained indicate that *CAS* may be part of a cell death pathway that is common for PE and DT as well as TNFα and -β, but distinct from other apoptosis-inducing agents like VP16 or staurosporine. This pathway is also different from apoptosis induced by inhibition of protein synthesis by cycloheximide. Cycloheximide mediated inhibition of protein synthesis can induce apoptosis under certain experimental conditions, but cycloheximide can also delay or inhibit apoptosis (Nakajima et al., 1994; Gong et al., 1993; Mizumoto et al., 1994; Chow, 1995). It appears that protein synthesis inhibition is not important per se but the means by which protein synthesis is inhibited is important; e.g., cycloheximide inactivates ribosomes while the toxins PE and DT inactivate elongation factor EF2.

The existence of a common pathway for DT, PE, and TNF mediated apoptosis has been previously suggested based on studies of the synergistic cytotoxic effects of toxins and TNF (Morimoto & Bonavida, 1992). But little is known about this pathway, and how the ADP-ribosylating toxins DT and PE induce apoptosis. There is some evidence that suggests a role of *CAS* in cell proliferation. *CAS* is homologous to the yeast *CSE1* gene which is involved in cell division and associated with B-type cyclin degradation (Irniger et al., 1995). *CAS* is highly expressed in proliferating cells. *CAS* is induced when cells are stimulated to proliferate, and *CAS* levels fall in growth arrested cells (Brinkmann et al., 1995a). What role does *CAS* play in the toxin/TNF apoptosis pathway, and what is the link between a function of *CAS* in cell proliferation and apoptosis? So far, our data do not provide a clear answer to this question. However, the intracellular localization of CAS on microtubules and the mitotic spindle (Scherf et al., 1996) and a putative recognition site for MAP-kinase (MEK) in CAS (Brinkmann et al., 1995a) raises the possibility that *CAS* is involved in the MAP-kinase regulation system (Seger & Krebs, 1995). This could explain a simultaneous role of *CAS* in proliferation and TNF mediated apoptosis because the MAP-kinase system regulates not only cell proliferation but also TNF mediated and neuronal apoptosis (Guy et al., 1995; Gupta et al., 1995; Xia et al., 1995). Analysis of the possible role of CAS as a substrate for MAP-kinases is in progress to evaluate this hypothesis.

CAS is the human homologue to a yeast chromosome segregation gene, and it has been shown that chromosome segregation genes can be cell cycle check points (Hartwell & Kastan, 1994; Nugroho & Mendenhall, 1994). Furthermore, involvement of *CSE1* in B-type cyclin degradation has already been demonstrated (Irniger et al., 1995). It is feasible that genes like *CAS* or *CSE1* may be "switch points" to decide whether a cell should proliferate or undergo apoptosis. Genes that affect apoptosis as well as cell proliferation, like *MYC*, *P53*, or *BCL-2*, often participate in cancer development (Hartwell & Kastan, 1994; Hermeking & Erick, 1994; Nishioka & Welsh, 1994). We have recently mapped the *CAS* gene on human chromosome 20 in a region (q13) that often contains amplications in aggressive breast cancer, and we have found that the *CAS* gene is specifically amplified in BT474 breast cancer cells (Brinkmann et al., 1996). It is therefore possible that *CAS* has a role in some cancers.

ACKNOWLEDGMENT

We thank E. Lovelace for excellent cell culture assistance and Keith Webber for help with TNF binding assays. For M.G., this publication is a partial fulfillment of the requirements for a Ph.D. from George Washington University. We also thank R. Mann and A. Jackson for editorial assistance.

REFERENCES

Brinkmann, U., & Pastan, I. (1994) *Biochim. Biophys. Acta 1198*, 27−45.

Brinkmann, U., Pai, L. H., FitzGerald, D. J., Willingham, M., & Pastan, I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 8616−8620.

Brinkmann, U., Brinkmann, E., Gallo, M., & Pastan, I. (1995a) *Proc. Natl. Acad. Sci. U.S.A. 92*, 10427−10431.
Brinkmann, U., Brinkmann, E., & Pastan, I. (1995b) *Mol. Med. 1*, 206−216.
Brinkmann, U., Gallo, M., Polymeropoulos, M. H., & Pastan, I. (1996) *Genome Res. 6*, 187−194.
Carroll, S. F., & Collier, R. J. (1987) *J. Biol. Chem. 262*, 8707−8711.
Chang, M. P., Bramhall, J., Graves, S., Bonavida., B., & Wisniesky, B. J. (1989) *J. Biol. Chem. 264*, 15261−15267.
Chaudhary, V. K., Jinno, Y., FitzGerald, D. J., & Pastan, I. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 308−312.
Chow, S. C., Peters, I., & Orrenius, S. (1995) *Exp. Cell Res. 216*, 149−159.
Cohen, J. J. (1993) *Immunol. Today 14*, 126−130.
Compton, M. M. (1992) *Cancer Metastasis Rev. 11*, 105−119.
Cory, A. H., Owen, T. C., Barltrop, J. A., & Cory, J. G. (1991) *Cancer Commun. 3*, 207−212.
Fendrick, J. L., Iglewsky, W. J., Moehring, J. M., & Moehring , T. J. (1992) *Eur. J. Biochem. 205*, 25−31.
Gong, J., Li, X., & Darzynkiewicz, Z. (1993) *J. Cell Physiol. 157*, 263−270.
Gottesman, M. M., & Pastan, I. (1993) *Annu. Rev. Biochem. 62*, 385−427.
Gudkov, A. V., Zelnick, C. R., Kazarov, A. R., Thimmapaya, R., Suttle, D. P., Beck, W. T., & Roninson, I. B. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 3231−3235.
Gudkov, A. V, Kazarov, A. R., Thimmapaya, R., Axenovich, S. A., Mazo, I. A., & Roninson, I. B. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3744−3748.
Gupta, S., Campbell, D., Dérijard, B., & Davis, R. J. (1995) *Science 267*, 389−393.
Guy, G. R., Philp, R., & Tan, Y. H. (1995) *Eur. J. Biochem.* 229, 503−511.
Hartwell, L. H., & Kastan, M. B. (1994) *Science 266*, 1821−1828.
Hermeking, H., & Eick, D. (1994) *Science 265*, 2091−2093.
Hirt, B. (1967) *J. Mol. Biol. 26*, 365−369.
Irniger, S., Piatti, S., Michaelis, C., & Nasmyth, K. (1995) *Cell 81*, 269−277.
Kido, M., Miwatani, H., Kono, K., Uchida, T., & Okada, Y. (1991) *Cell Struct. Funct. 16*, 447−453
Kochi, S. K., & Collier, R. J. (1993) *Exp. Cell Res. 208*, 296−302.
Laurie, S. M., & Robbins, A. R. (1991) *J. Cell. Physiol. 147*, 215−223.
Lowe, S. W., Schmitt, M., Smith, S. W., Osborne, B. A., & Jacks, T. (1993) *Nature 362*, 847−849.
Maniatis, T., Sambrook, J., & Frisch, E. F. (1989) in *Molecular Cloning* (*A Laboratory Manual*), 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Mizumoto, K., Rothman, R. J., & Farber, J. L. (1994) *Mol. Pharmacol. 46*, 890−895.
Morimoto, H., & Bonavida, B. (1992) *J. Immunol. 149*, 2089−2094.
Munson, P. J., & Rodbard, D. (1983) *Methods Enzymol. 92*, 543−576.
Nakajima, M., Kashiwagi, K., Ohta, J., Furukawa, S., Hayashi, K., Kawashima, T., & Hayashi, Y. (1994) *Brain Res. 641*, 350−352.
Nishioka, W. K., & Welsh, R. M. (1994) *J. Exp. Med. 179*, 769−774.
Nugroho, T. T., & Mendenhall, M. D. (1994) *Mol. Cell. Biol. 14*, 3320−3328.
Ogryzko, V. V., Hirai, T. H., Shih, C. E., & Howard, B. H. (1994) *J. Virol. 68*, 3724−3732.
Pastan I., Chaudhary, V. K., & FitzGerald, D. J. (1992) *Annu. Rev. Biochem. 61*, 331−354.
Scherf, U., Pastan, I., Willingham M. C., & Brinkmann, U. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 2670−2674.
Schimke, R. T. (1988) *Cell 37*, 705−713.
Seger, R., & Krebs, E. G. (1995) *FASEB J. 9*, 726−735.
Sentman, C. L., Shutter, J. R., Hockenbery, D., Kanagawa, O., & Korsmeyer, S. J. (1991) *Cell 67*, 879−888.
Smith, C. A., Farrah, T., & Goodwin, R. G. (1994) *Cell 76*, 959−962.
Suda, T., Takahashi, T., Goldstein, P., & Nagata, S. (1993) *Cell 75*, 1169−1178.
Webber, K. O., Reiter., Y., Brinkmann, U., Kreitman, R. J., & Pastan, I. (1995) *Mol. Immunol. 32*, 249−258.
Williams, G. T., & Smith, C. A. (1993) *Cell 74*, 777−779.
Xia, Z., Dickens, M., Raingeaud, J., David, R. J., & Greenberg, M. E. (1995) *Science 270*, 1326−1331.
Xiao, Z., McGrew, J. T., Schroeder, A. J., & Fitzgerald-Hayes, M. (1993) *Mol. Cell. Biol. 13*, 4691−4702.

BI952829+