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INTRODUCTION

The purpose of the study. Most cells, including cancer cells resistant to available drugs, retain a suicide machinery that can kill the cell within an hour through apoptosis. A current view is that apoptosis is executed by a proteolytic cascade that consists of a family of proteases known as caspases, their co-factors and inhibitors. The cascade can be initiated at several points by activating initiator caspases. Apoptosis is mediated by two pathways, intrinsic, which requires caspase-9, and extrinsic, which requires caspase-8. Caspase-9 is activated by a series of reactions that require cytochrome c, which is released from the mitochondria following a number of stimuli summarily referred to as cytotoxic stress. Caspase-8 is activated through transmembrane proteins known as death receptors, which are activated by binding to their ligands. Caspase-8 and -9, known as initiator caspases, then activate executioner caspases, such as caspase-3 and caspase-7, which disassemble the cell by processing a number of proteins. Because each initiator caspase mediates a subset of cytotoxic signals, a prerequisite to the understanding how a particular signal induces apoptosis is to identify the initiator caspase it activates, and learn how this activation occurs.

BODY OF THE REPORT

This study began with our observation that a prostate cancer cell line DU145 treated with etoposide, a chemotherapeutic that induces DNA damage, was undergoing apoptosis manifested by characteristic morphological changes and increased binding to Annexin V, a marker of apoptotic cells (Figure 1). However, this death was accompanied by barely detectable caspase processing (Figure 2), which made us suspect that DU145 cells die by an "unconventional" pathway. Inhibitors of caspases ZVAD-FMK (Figure 3) and viral protein p35 (Figure 4), prevented apoptosis, as did Bcl-2 (Figure 4), which inhibits cytochrome c release from mitochondria. These results were consistent with the notion that apoptosis in DU145 cells requires caspases. However, we found that crmA, a viral inhibitor of caspase-8, or caspase-9 dominant negative mutant, which prevents activation of this caspase, had no effect on apoptosis in DU145 cells if used separately or in combination (Figure 5). This observation indicated that these two caspases are not required for apoptosis induced by chemotherapy in this cell line. To reconcile these observations we hypothesized that apoptosis in DU145 is mediated by an unidentified caspase that we named caspase-P, which we proposed to identify.

The proposed approaches. To identify caspase-P we decided to exploit the observation that p35 prevents apoptosis and the fact that this protein forms highly stable complexes with inhibited caspases. We developed an approach to purify active caspases by isolating the p35-caspase complexes and, in case this approach fails, also proposed to explore the requirement for known caspases and putative caspases that could be identified by the Human Genome Sequencing Project, which was moving towards completion at the time our proposal was submitted.

A change in strategy. Two events that occurred during the first year of this study modified our experimental strategy. The first event, which happened earlier than we expected, was the announcement that the sequence of the human genome was largely completed. The reported sequence provided no additional caspase family candidates, indicating that the identified members constitute the complete set, although this still left open the possibility that structurally distinct proteins may have caspase activity. The second development was the truly revolutionary discovery that RNA interference (RNAi) can be used to silence gene expression in cultured mammalian cells. Considering these developments, we decided to exploit small interfering RNA (siRNA) as a tool to systematically silence expression of caspases in DU145 cells. This approach appeared to require much less effort and resources, and promised to provide substantial "side" benefits, such as the capability to verify our preliminary results obtained with caspase inhibitors, and to develop basic tools that can be used by us and others to analyze molecular mechanisms in human tumor cells.

Caspase-2 is required for apoptosis. As a first RNAi target we decided to use caspase-2 because no other tools to determine a requirement for this caspase in a cell line were available. Caspase-2 is ubiquitously expressed but had no clearly defined function at the time. Although some studies implicated this caspase in apoptosis, mice deficient in caspase-2 show no clear defects in cell death, except in the ovary.

We designed a siRNA to caspase-2 but then realized that we were not ready to test it in DU145 cells. The transfection efficiency, which was about 10%, was insufficient to monitor caspase-2 expression in total cell lysates, and we had no tools to monitor the expression in individual cells. Therefore, we tested the siRNA on human fibroblasts transformed with the adenoviral oncogene E1A (IE cells), which is a model system that we routinely use and can transfect with greater than 90% efficiency. Our primary goal was to determine whether the siRNA silences the expression of its target, and we found that it did. To our surprise, we also found that silencing caspase-2 expression prevented apoptosis induced by etoposide, UV, and cisplatin (Lassus et al., 2002a). The subsequent experiments revealed that silencing prevented release from mitochondria of cytochrome c, a protein required for activation of caspase-9. Our surprise was understandable considering the common doubts that caspase-2 is involved in apoptosis and the view that cytochrome c release is caspase-independent. We expanded our finding to two other human cancer cell lines (Lassus et al., 2002a), an observation that indicated that the mechanism we identified is not limited to our experimental model.

Although clearly a digression from the initial goal of the planned research, the study of the requirement for caspase-2 in apoptosis contributed to our understanding of apoptosis, provided new tools and approaches to study it. Taken at face value, our findings indicate that in response to chemotherapy, mitochondria are amplifiers of caspase activity rather than initiators of caspase activation, as it has been widely believed. Our study also raised the need to understand the regulation of caspase-2, which was practically unknown, resulting in studies that provided some insights in how this caspase is activated (Read et al., 2002; Tinel and Tschopp, 2004). Besides identifying a new pathway of apoptosis, our study also demonstrated that RNAi can be successfully used to study the requirement

of a gene in human cancer cells, thus eliminating the necessity to extrapolate results obtained in genetically modified mice. Our study also introduced an approach to verify the specificity of RNAi. This approach, which is now widely used, involves ectopic expression of the cDNA for the gene targeted by RNAi, in which the sequence cognate to the siRNA is modified by silent mutations to prevent destruction by RNAi (Lassus et al., 2002b).

Recently we made an observation that led us to revisit our conclusion that caspase-2 is required for apoptosis. We identified one siRNA that silenced caspase-2 expression, as detected by immunoblotting, but did not prevent cell death. We have been considering several explanations for this finding. One is that our published results were due to either a general non-specific effect, such as the interferon response. We found no indications of the interferon response, such as cell cycle arrest or phosphorylation of the translation initiation factor eIF2 α , and determined that both silencing and the anti-apoptotic effect of the siRNA are sequence specific. Another possibility is that the published siRNA silences more than one gene. We have been investigating this possibility with extensive help from the experts in RNAi and bioinformatics, but have identified no targets other than caspase-2. It also is possible that caspase-2 is expressed as more than one splicing variant and that their relative abundance or interaction determines cell viability. This possibility is consistent with the reports of alternative caspase-2 splicing (Droin et al., 2001; Logette et al., 2003). We are currently investigating in detail caspase-2 expression on the protein and mRNA level and testing whether any of the isoforms are silenced differently by the siRNAs to caspase-2. Fortunately, as a part of this study, we have made several polyclonal and monoclonal antibodies to caspase-2, which should facilitate the analysis. We have been communicating our findings to the scientific community through meetings and seminars. We feel that full understanding of our observations, whatever the mechanism that makes cells drug-resistant is, will help to understand better how cell viability is regulated.

The successful implementation of the RNAi approach encouraged us to use it to systematically analyze the role of known caspases in DU145 cells. As the first step, we optimized transfection of siRNA in DU145 cells. To monitor the effect of variables in the transfection conditions we used a siRNA to Smac, a mitochondrial protein that can be easily detected by immunofluorescence with an antibody that we developed. We now use this approach whenever we need to develop transfection conditions for a cell line or to train a new researcher in using siRNA.

Requirement for caspases in apoptosis in DU145 cells. Once we increased the transfection efficiency to 70%, we were ready to test the requirement of caspases in DU145 cells. We first tested whether caspase-2 is required for apoptosis in DU145 cells and found that it was not (Figure 6), consistent with our findings that the mechanisms of stress-induced death of these cells is different from that observed in other cell lines.

We also analyzed the requirement for Apaf-1, and caspases 4, 5, 6, and 7, the proteins whose requirement we could not test before the RNAi approach became available. We found that silencing expression of either caspase-6 or caspase-7 had no effect on cell

death (Figure 6). Because we had no tools to monitor expression of the caspase-4 and 5 proteins, we monitored the efficiency of silencing by ectopically expressing these proteins as fusions with the myc epitope tag (Figure 7). We found that siRNAs to these caspases also did not prevent apoptosis in DU145 cells. The failure of Apaf-1 silencing to prevent apoptosis was consistent with our previous finding that caspase-9 dominant negative mutant also had no effect, thus providing an additional piece of evidence that the caspase-9 pathway is not required for drug-induced apoptosis in these cells. The finding that silencing caspases 4 and 5, which function is unknown, also had no effect, was consistent with our preliminary observation that crmA, a viral caspase inhibitor that was reported to inhibit caspase-4 and caspase-5 weakly *in vitro*, had no effect in this cell line.

Given that at this stage none of the tested caspases appeared to be required for apoptosis, we considered two explanations. One was that two or more caspases are functioning simultaneously, in which case silencing expression of one of them may have little effect. For example, caspase-9 and caspase-2 have been shown to be compensatory in certain cases (Troy et al., 2001). The second possibility was that caspase-P is a protein that has caspase activity but is distinct from caspases structurally, which would imply that we would not find caspase-P by testing the requirement for the members of the caspase family.

Silencing of caspases -7 and -8 inhibits apoptosis. To test whether several caspases function simultaneously, we attempted to develop an approach to silence expression of more than one gene by transfecting several siRNAs. Unfortunately, this approach failed. As I understand, many laboratories have experienced difficulties with this approach for unknown reasons. As an alternative, we decided to silence expression of individual caspases that express crmA (DU-crmA), an inhibitor of caspases 1, 8, and perhaps, 4 and 5, and cells that express the caspase-9 dominant negative mutant, which inhibits activation of this caspase. We have found that a siRNA to caspase-7 decreased the rate of apoptosis in DU-crmA, but not in the parental cell line or the cells that express C9DN (Figure 8). This finding was somewhat surprising, considering that caspase-7 has been considered an executioner caspase that functions redundantly to caspase-3. However, the results were reproducible in multiple experiments using two independent DU-crmA lines. We considered several explanations for our observation (Figure 9).

Before exploring these possibilities, we decided to verify that the observed effect of crmA and siRNA to caspase-7 is due to silencing of the intended targets. CrmA strongly inhibits both caspases-8 and -1 and has some activity for caspases-4 and -5 (Garcia-Calvo et al., 1998; Stennicke et al., 2002). Since the role of caspase-8 in apoptosis is well established, we first investigated whether inhibition of this caspase is responsible for the effect of crmA in our system. Our approach was to test whether the effect of CrmA can be reproduced by using a caspase-8 dominant negative (C8DN) mutant that is a catalytically inactive caspase-8, or FlipL (Irmeler et al., 1997), a cellular protein that functions as C8DN. We found that either FlipL or C8DN could replace crmA in our assay (Figure 10), consistent with the notion that caspase-8 was the target of crmA responsible for the observed effect.

To verify that the effect of the caspase-7 siRNA (siC7.1) is due to silencing of its intended target, we are using two approaches. We are testing whether another siRNA to caspase-7 (siC7.2) has the same effect, and are investigating whether the effect of silencing can be prevented by ectopic expression of caspase-7 using the approach that we developed investigating the requirement of caspase-2 in apoptosis. In two preliminary experiments, the siC7.2 inhibited apoptosis in DU-crmA and DU-FlipL cells, but not as efficiently as siC7.1, perhaps because siC7.2 is not as efficient at silencing expression of caspase-7 (Figure 10). To implement the second approach, we are making DU145 cells that express caspase-7 that is not silenced by siC7.1.

siRNA to caspase-5 inhibits apoptosis in DU145 cells. To confirm that the phenotype that we observe in DU-145 cells is stable in time, and to make sure our conclusions are based on strong foundation, we repeated all our critical experiments. We made new derivatives of DU145 cells that express crmA, caspase-9 dominant negative mutant, both of these proteins together, and bcl-2, and re-tested all siRNAs to caspases. We also confirmed that transient transfection of p35 prevents apoptosis, and expanded the experiment by finding that a p35 mutant that is unable to inhibit caspases fails to prevent apoptosis in DU-145 cells. We found two differences with our previous results. In contrast to our previous studies we did observe some caspase processing, including caspase-7 and caspase-8 (Figure 11). Another difference was in a requirement for caspase-5. While doing these experiments, we analyzed cell death not only by binding to Annexin V, which has been our main assay because of its convenience, but also by scoring cells with apoptotic nuclear morphology. While doing these experiments, we found that silencing expression of caspase-5 prevented apoptosis as scored by nuclear morphology (Figure 12). We repeated this experiment four times with reproducible results, which made us think that the original experiment that found no effect of the caspase-5 siRNA were flawed, either because of our inexperience, the lack of controls, or because it was not repeated to make the data sufficiently reliable.

To test whether the observed effect is due to silencing of caspase-5 rather than some other effect of the siRNA, we are using several approaches. We are testing whether two other caspase-5 siRNAs, which we have designed, inhibit apoptosis. We are also testing whether we can rescue the effect of silencing by ectopic expression of caspase-5 (Lassus et al., 2002b).

If caspase-5 is activated during apoptosis, this caspase is likely to be processed in apoptotic cells. To test whether this is true, we are using two approaches. One is to monitor processing of caspase-5 that is ectopically expressed as a fusion with the myc epitope tag, another is to detect endogenous caspase-5. We found no reliable commercial antibody to caspase-5 and are making polyclonal or monoclonal antibodies to this caspase.

Caspase-5 is close structurally to human caspases 1 and 4 and mouse caspases 11 and 12, which were all implicated in inflammation. Consistent with this similarity, caspase-5 has been implicated in inflammation response (Lin et al., 2000; Martinon et al., 2002). At the same time, frameshift mutations in caspase-5 have been observed in lung, colorectal,

endometrial, and gastrointestinal cancers as well as T-cell lymphoblastic lymphomas or leukemias (Hosomi et al., 2003; Schwartz et al., 1999; Scott et al., 2003; Takeuchi et al., 2003; Trojan et al., 2004). An explanation for these mutations comes from the finding that caspase-5 has a polyadenine A(10) tract that is susceptible to microsatellite instability (MSI) in cancer cells. These observations suggest that caspase-5 might be selected against in cancers due to its role either in inflammation response or, perhaps, in apoptosis. The second possibility is consistent with the fact that caspase-5 has a CARD domain, which is characteristic of other initiator caspases such as caspase-9. Another link may be related to homology of caspase-5 to the mouse caspase-12, which is localized at the endoplasmic reticulum (ER), and thought to mediate ER-stress induced cell death (Nakagawa et al., 2000). Overall, although there is no direct evidence that caspase-5 participates in apoptosis, the evidence that it does not is also unavailable.

The model that we are testing is that caspase-5 is caspase-P, which activates caspase-7 and caspase-8 (Figure 13). As Bcl-2 inhibits this cell death but cytochrome c is not released and the caspase-9 is not required, the model implies that Bcl-2 functions by regulating caspase-5.

Overall, our observations that cell can die by some unidentified caspase-dependent pathway are remarkably similar to that made by Andreas Strasser's group (Marsden et al., 2004; Marsden et al., 2002), who observed that apoptosis in a reconstituted haematopoietic mouse model does not require Apaf-1, caspase-9, or caspase-2, and is inhibited by Bcl-2. The difference in our observations is that we do not observe release of cytochrome c during apoptosis. These similarities make it likely that the pathway that we observe is not limited to DU145 cells. We will continue our research to identify this pathway.

KEY RESEARCH ACCOMPLISHMENTS

We proposed a role for caspase-2 in apoptosis, which modified existing models of apoptotic pathways. We advanced towards identifying a new pathway of apoptosis, and developed a set of tools and approaches that have been used by other researchers to investigate cancer biology.

REPORTABLE OUTCOMES

Publications:

Lassus, P., Opitz-Araya, X., Lazebnik, Y. Requirement for caspase-2 in stress-induced apoptosis prior to mitochondrial permeabilization. *Science*, in press, 2002.

Lassus, P., Rodriguez, J., Lazebnik, Y. Confirming specificity of RNAi. *Science's STKE*, in press, 2002.

The results of the study were reported at the following scientific meetings and invited seminars.

Meetings:

The Servier International Conference "Understanding the molecular basis of cancer for therapeutic benefit", France

February 13-17, 2002. AACR Special Conference on Apoptosis and Cancer, Hawaii

March 21-22, 2002. The National Institute of Child Health and Development workshop on apoptosis in the reproductive system, Bethesda, MD. The title "Mechanisms of apoptosis".

April 18-21, 2002. EMBO Workshop on Nuclear Structure and Function, Prague

February 13-16, 2003. The 15th Lorne Cancer Conference, Lorne, Australia. Title "Oncogenic Transformation as an Activator of Apoptosis".

May 15-18, 2003. The Deutsche Forschungsgemeinschaft (DFG) annual meeting of the "Hinterzarter Kreis" for Cancer Research, Cadenabbia, Italy. Title "Oncogenic Transformation as an Activator of Apoptosis".

Invited Seminars:

March 13, 2002. University of Vermont, Department of Pathology, Burlington, VT. The title "Oncogenes as a Trojan horse". Hosted by Dr. Ralph Budd.

October 2, 2002. University of Connecticut Center for Vascular Biology Seminar Series. Farmington, CT. Title "Oncogenic Transformation as a Cause of Cell Death". Hosted by Dr. Henry Furneaux.

October 22-23, 2002. M.D. Anderson Cancer Center, the Blaffer Series Seminars. Houston, TX. Title "Oncogenic Transformation as an Activator of Apoptosis" Hosted by Dr. Ryuji Kobayashi.

December 14, 2002. Karolinska Institutet, Stockholm, Sweden. Title "Biochemical mechanisms of apoptosis". Hosted by Dr. Sten Orrenius.

January 6, 2003. Tufts University, Department of Physiology, Boston, MA. Title "Apoptosis, cancer, and martial arts". Hosted by Dr. Irwin Arias.

March 14, 2003. National Cancer Institute, Genome Structure and Function Section in the Laboratory of Biosystems and Cancer. "Oncogenic transformation as a cause of apoptosis". Hosted by Dr. Vladimir Larionov.

March 17, 2003. Vanderbilt University, Department of Biochemistry, Nashville, TN. Title "Proteases in apoptosis". Hosted by Dr. Graham Carpenter.

April 15, 2003. James Graham Brown Cancer Center, University of Louisville, Louisville, Kentucky. Title "Cell death, martial arts, and a radio". Hosted by Dr. Jason Chesney.

May 22, 2003. University of Medicine and Dentistry of New Jersey, Department of Biochemistry. Newark, NJ. Title "Oncogenic transformation as a cause of apoptosis". Hosted by Dr. Raymond Birge.

April 27, 2004. Institute of Cancer Biology, Danish Cancer Society, Copenhagen, Denmark. Title: "Oncogenic transformation as a cause of apoptosis". Hosted by Marja Jaattela.

Reagents produced during the course of the study:

My laboratory's policy is to provide all published reagents to academic researchers with no obligations on their part except mentioning the source of the reagents in publications.

Cell lines:

All cell lines were made using retroviral transduction.

DU145-crmA
DU145-C9DN
DU145-crmA/C9DN
DU145-C8DN
DU145-FlipL

IE-caspase-2 (human fibroblasts transformed with E1A and expressing caspase-2)
IE-caspase-2si (caspase-2 resistant to siRNA)
IE-caspase-2Cys (catalytically inactive caspase-2)

The cell lines currently in production:

DU145-Caspase-5 MycFlag
DU145-Caspase-5 Cys (catalytically inactive caspase-5)
DU145-Caspase-5si (caspase-5 resistant to siRNA)
IE-Caspase-5 MycFlag
IE-Caspase-5 Cys (catalytically inactive caspase-5)
IE-Caspase-5si (caspase-5 resistant to siRNA)

siRNA:

Functional siRNA to the following genes: Apaf-1, caspase-2, caspase-4, caspase-5, caspase-6, caspase-7, cytochrome c, Smac

Antibodies:

Monoclonal and polyclonal antibodies to caspase-2.
Antibodies to caspase-5 are in production.

CONCLUSIONS

Our systematic approach to chemotherapy-induced apoptosis helped us to advance the understanding of this process and to develop new tools to analyze mechanisms of cell death in tumor cells. Our proposal that a new pathway of apoptosis controls cell viability was confirmed although we still need to identify components of this pathway. In addition, our research stimulated research in other laboratories to understand activation of caspase-2 and by providing examples of using RNAi to study cell viability in human tumor cells.

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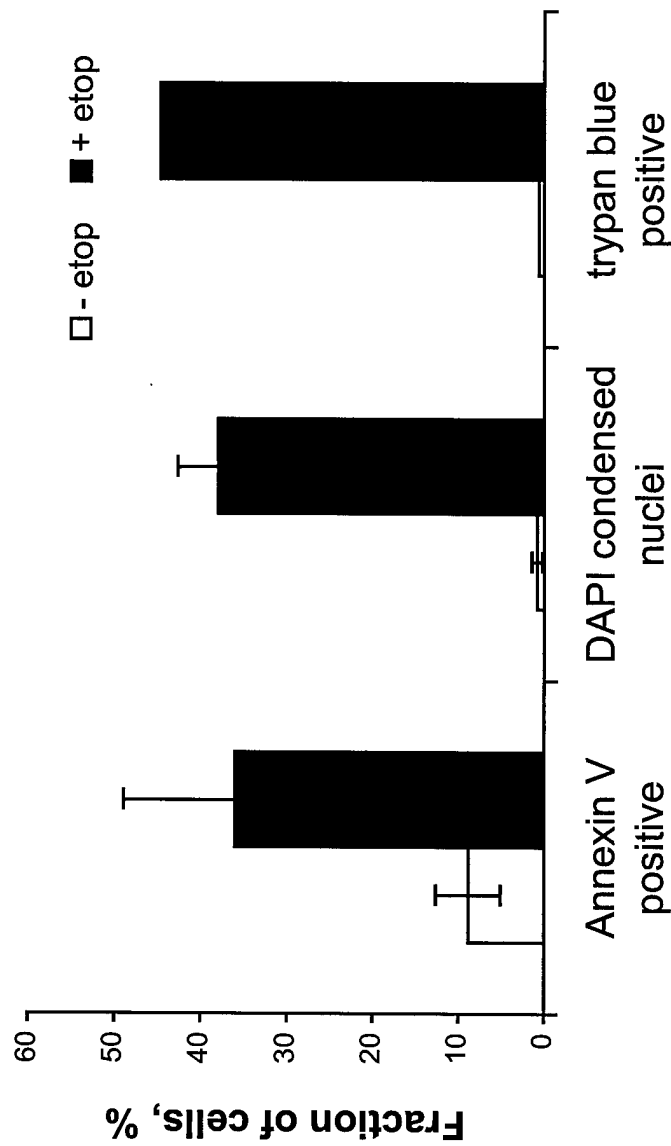


Figure 1. Etoposide induces cell death in DU145 cells that is associated with markers of apoptosis. Cells were treated for 30 hours with 100 μ M etoposide, collected, and the rate of apoptosis evaluated by scoring cells that bind Annexin V-FITC, have condensed chromatin, as revealed by staining with DAPI. The rate of total death was measured by scoring cells that fail to exclude Trypan blue .

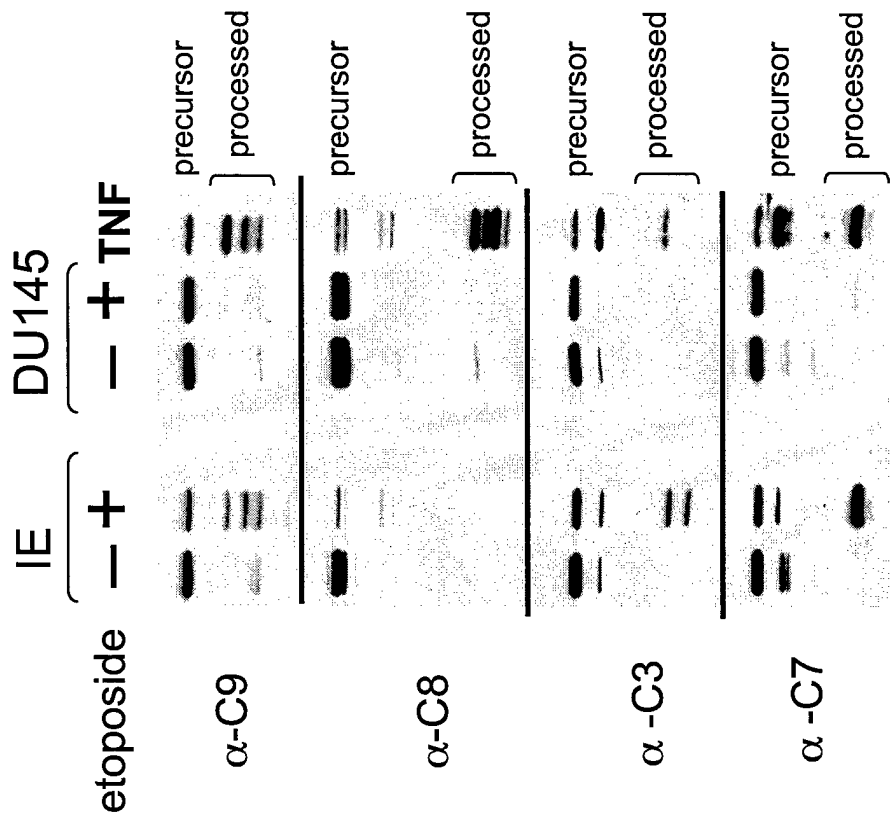


Figure 2. Processed caspases are easily detectable in apoptotic IE but not apoptotic DU145 cells. The rate of apoptosis in both cells was about 60%.

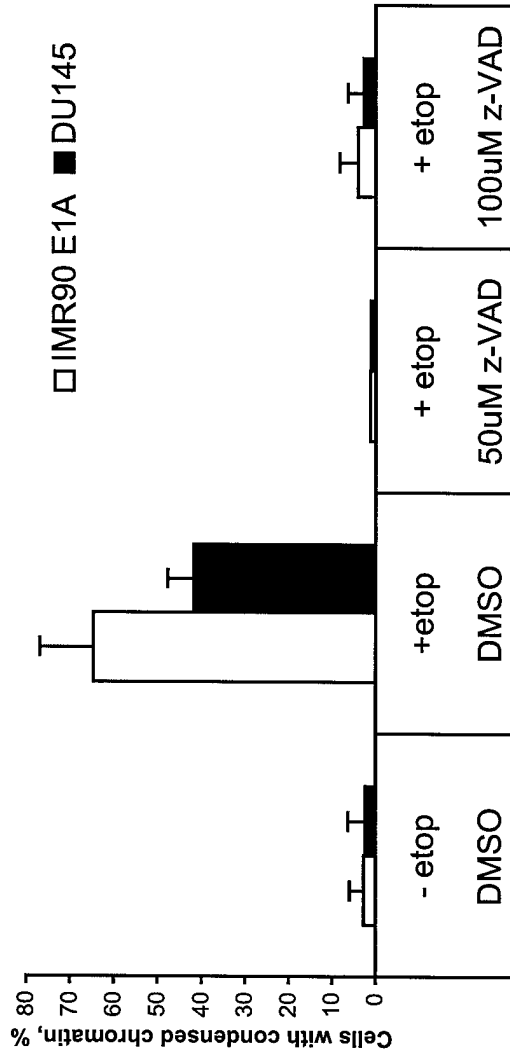


Figure 3. Apoptosis in DU145 cells is prevented by a peptide caspase inhibitor zVAD-FMK. DU145 or IMR90 E1A cells were treated for 2 hours with either 50 μM or 100 μM zVAD-FMK and then treated for 30 hours with 100 μM etoposide. The cells were collected, stained with DAPI and the rate of apoptosis evaluated by scoring cells with condensed chromatin.

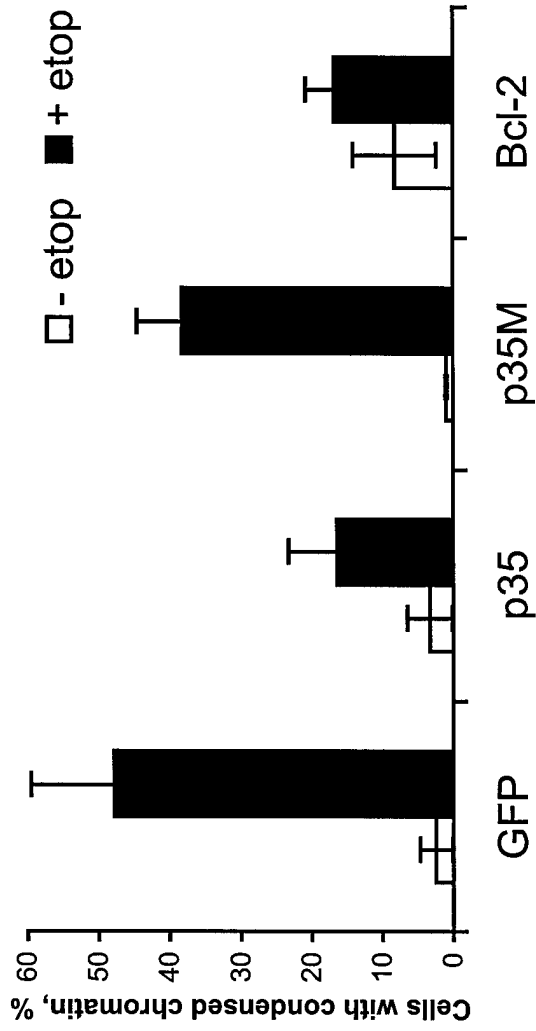


Figure 4. Apoptosis in DU145 cells is prevented by Bcl-2, p35, but not by a p35 mutant that fails to inhibit caspases. P35, P35 mutant, or Bcl-2 were transiently transfected with GFP and then treated with 100 μ M etoposide for 30 hours. The rate of apoptosis was measured as described in legend for Figure 3.

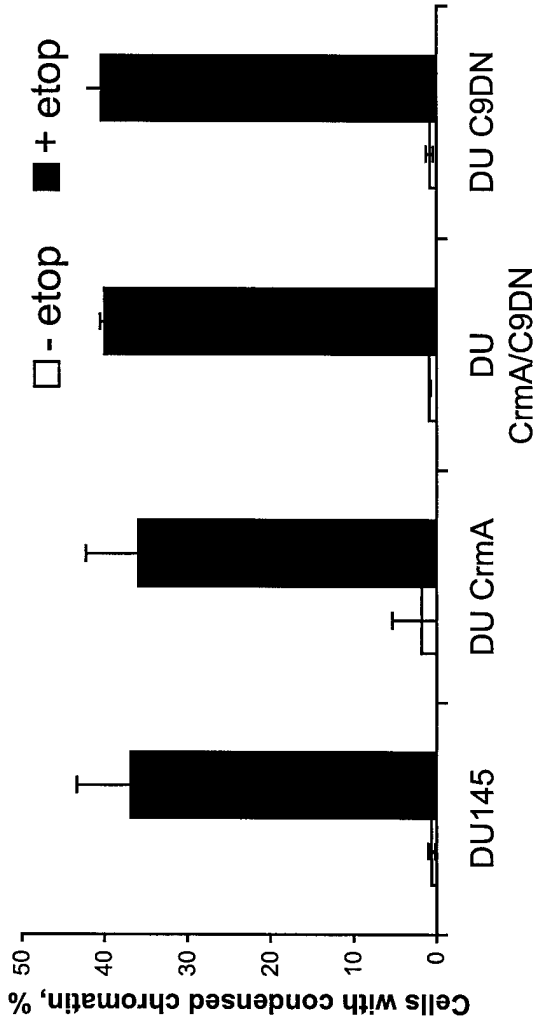


Figure 5. Apoptosis in DU145 cells is not prevented by CrmA, C9DN or both. DU145 cells, DU145 expressing crmA, C9DN, or both were treated with 100 μM etoposide for 30 hours and the rate of apoptosis was measured as described in legend for Figure 3. **A.** The rate of cell death. **B.** Verification of expression for C9DN and CrmA. (*) indicates non-specific band

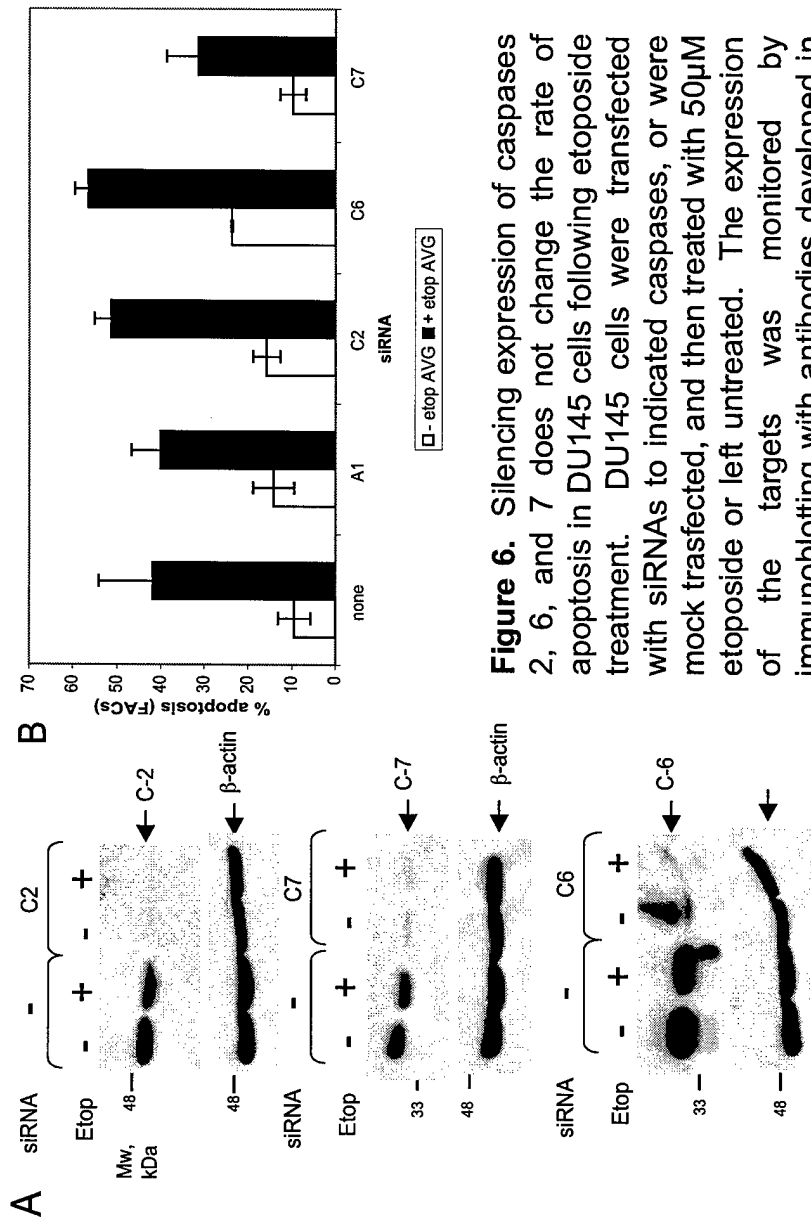


Figure 6. Silencing expression of caspases 2, 6, and 7 does not change the rate of apoptosis in DU145 cells following etoposide treatment. DU145 cells were transfected with siRNAs to indicated caspases, or were mock transfected, and then treated with 50 μ M etoposide or left untreated. The expression of the targets was monitored by immunoblotting with antibodies developed in our laboratory (**A**). The rate of apoptosis was determined by staining with Annexin V-FITC with subsequent detection by flow cytometry (**B**).

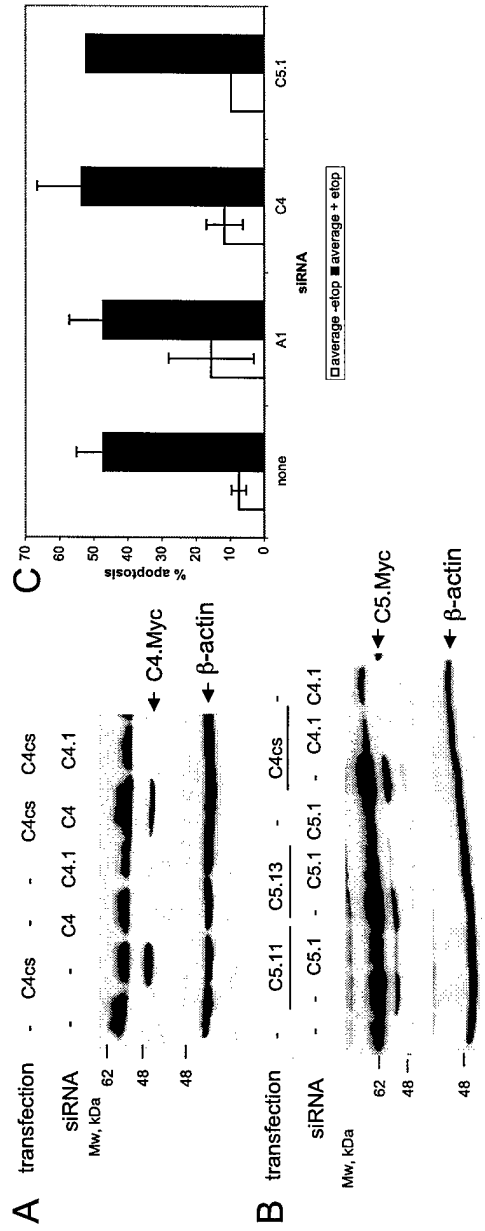


Figure 7. Silencing expression of caspases 4 and 5 has no effect of etoposide-induced apoptosis in DU145 cells. Because we found no reliable antibodies to caspases 4 and 5, we tested the efficiency of siRNA to these caspases by using ectopically expressed fusion of caspases 4 and 5 with a Myc epitope tag. We identified siRNAs to both caspases that efficiently inhibited expression of caspase-4-myc and caspase-5-myc (A, B). Our experience, and current knowledge about RNAi indicated that the endogenous target of an siRNA is silenced if the ectopically expressed protein is silenced. Therefore, we assumed that the siRNAs that we identified prevent expression of endogenous caspase-4 and caspase-5. We found no effect of this silencing on apoptosis, as detected by Annexin V staining with subsequent detection by flow cytometry (B). The experiments were done as described in Figure 2.

A

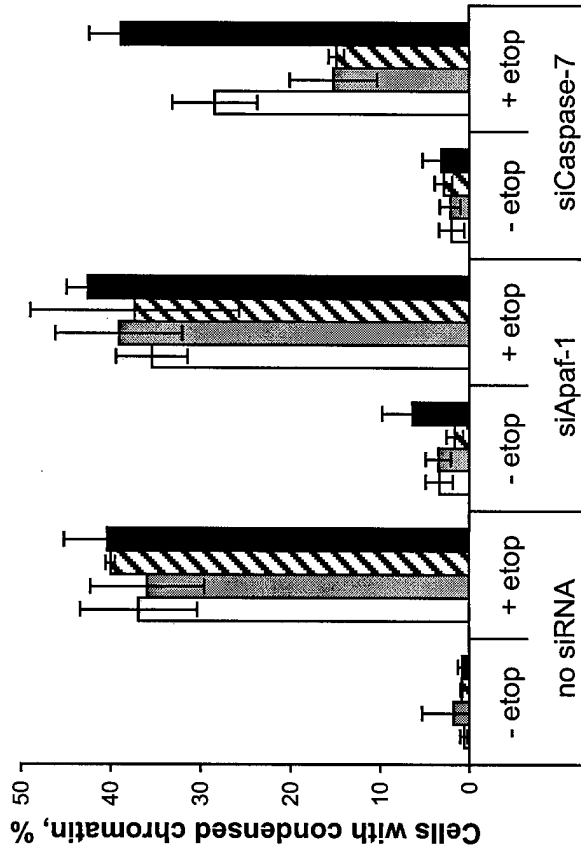
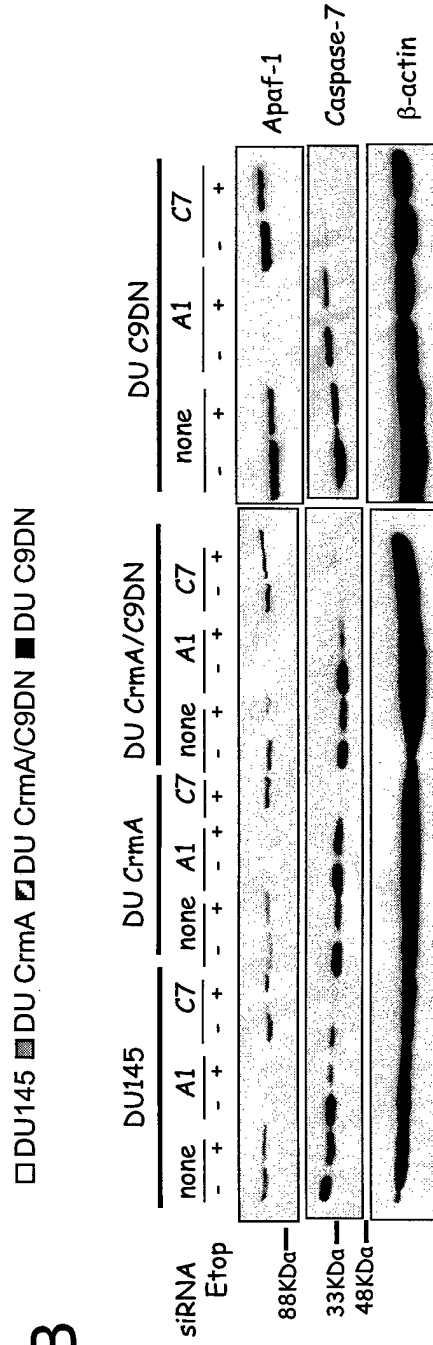


Figure 8. siRNA to caspase-7 inhibits apoptosis only in cells that express crmA. DU145 cell lines that express crmA, C9DN or both were transfected with siRNAs to caspase-7 or Apaf-1, treated with 100 μ M etoposide for 30 hours and the rate of apoptosis was measured as described in legend for Figure 3.

B



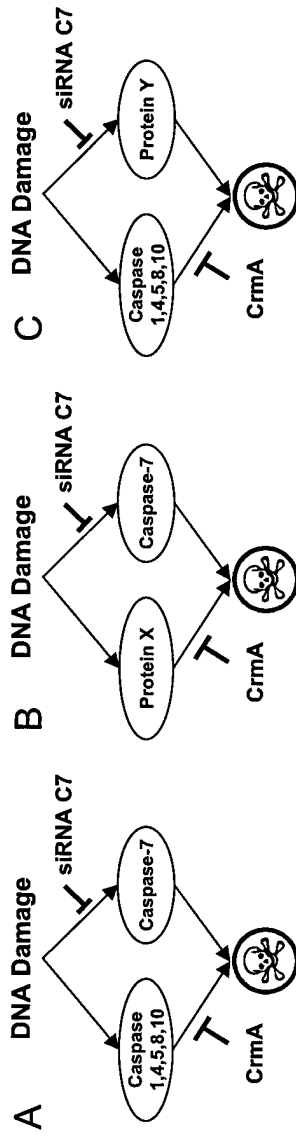


Figure 9. Models explaining the observation that crmA and the siRNA to caspase-7 synergize to prevent apoptosis in DU145 cells. All models assume that DNA damage induces more than one pathway. **A.** Both crmA and the siRNA eliminate the intended targets. This possibility implies that one of the caspases inhibitable by crmA is involved, which can be tested by silencing their expression one by one. **B.** CrmA inhibits a new target. This possibility will become plausible if silencing expression of known crmA targets will not mimic the effect of crmA. To find the protein inhibited by crmA, we will attempt to isolate molecules bound to this caspase inhibitor. **C.** siRNA to caspase-7 has an effect other than silencing of caspase-7 expression (an "off-target" effect). We are testing this possibility by investigating whether ectopic expression of caspase-7 can rescue the effect of the siRNA.

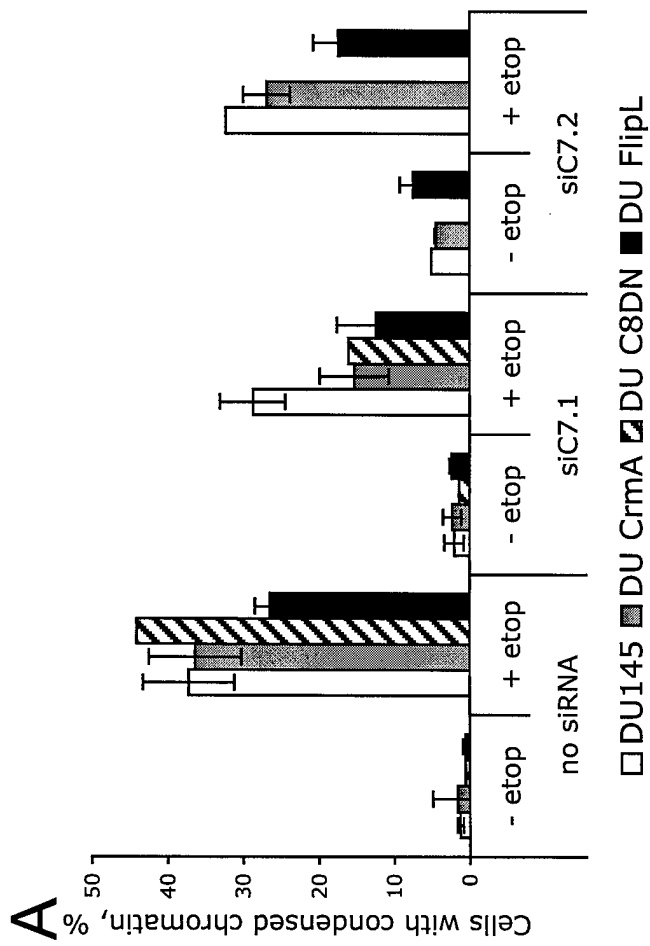
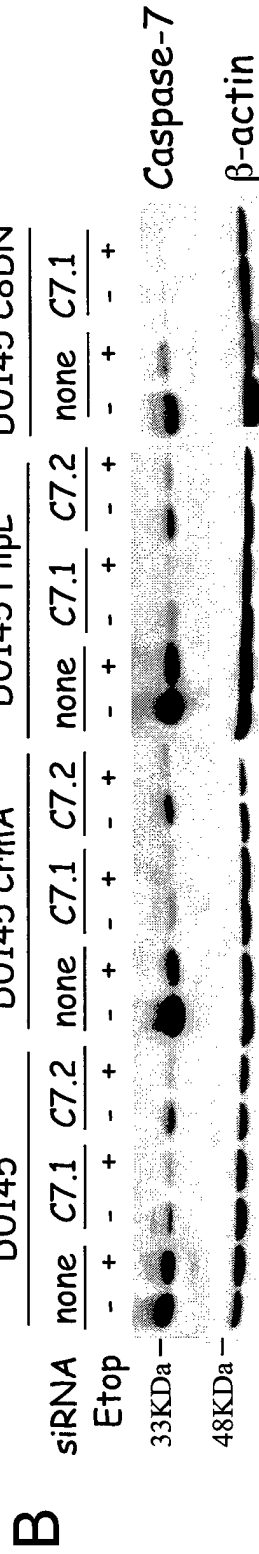


Figure 10. The inhibitors of caspase-8 activation caspase-8 dominant negative or FlipL can substitute crmA to inhibit apoptosis in cells treated with siRNAs to caspase-7. The experiments were performed as described in legend for Figure 8.



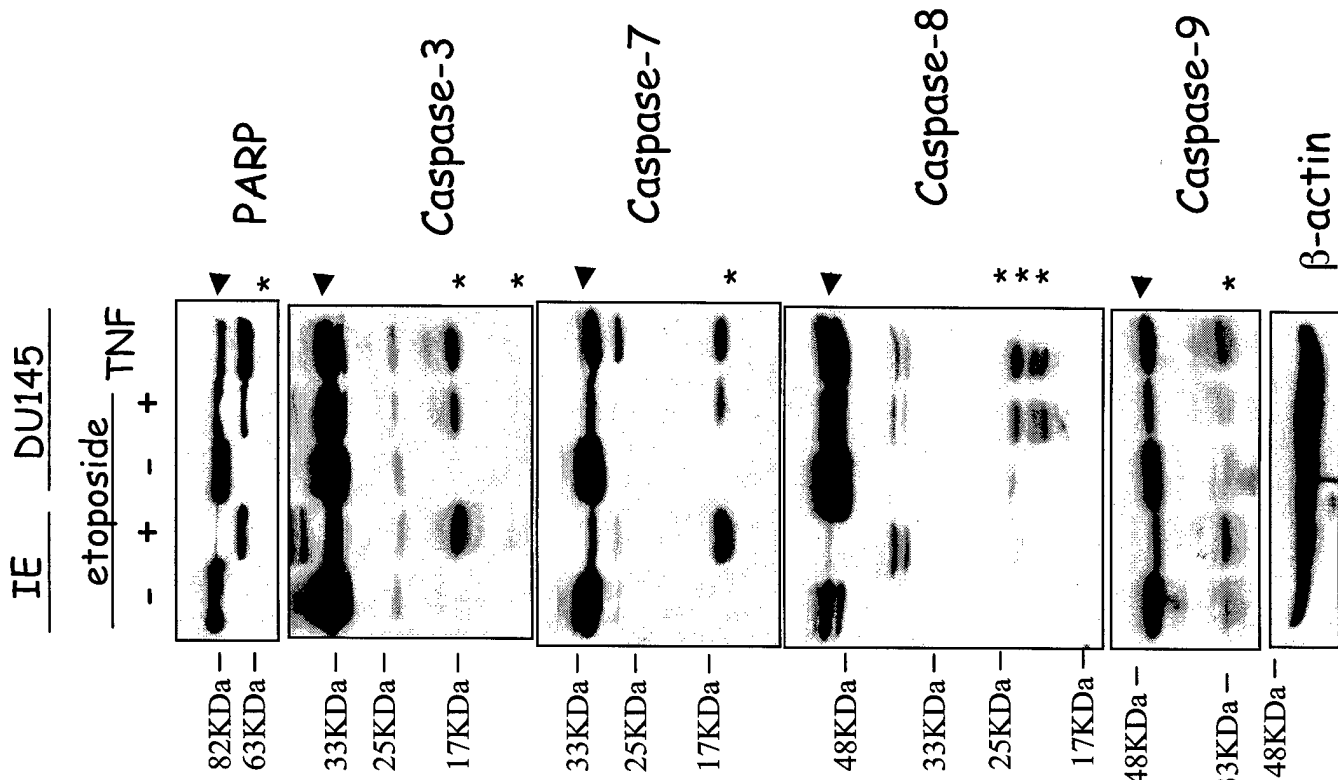


Figure 11. Processing of caspases in DU145 and IE cells treated with etoposide. Arrowheads indicate unprocessed form and stars processed forms.

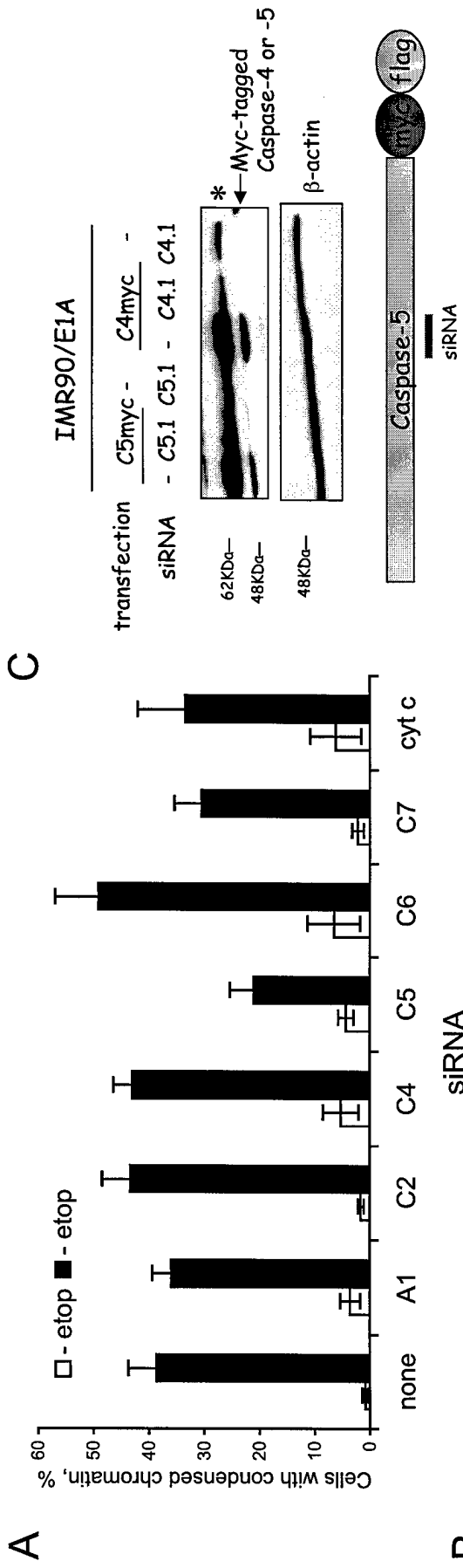


Figure 12. siRNA to caspase-5 inhibits apoptosis in DU145 cells (A). Detection of endogenous proteins in cells transfected with siRNA (B). Detection of silencing caspase-4 and caspase-5 by monitoring ectopically expressed proteins fused with the Myc epitope tag. (*) indicates a non-specific band (C). Detection of cytochrome c expression (D).

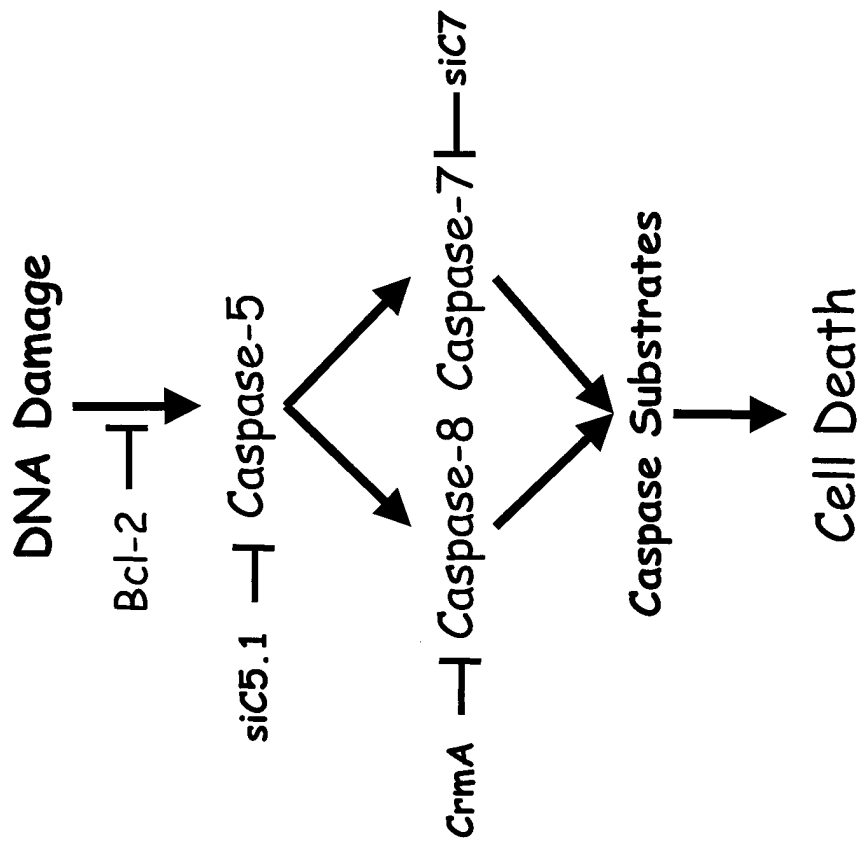


Figure 13. A model for cell death in DU145 cells

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diffusion of secreted substances and promotes paracrine interactions within the islet, where β , α , and δ cells tend to be segregated (14).

Our data suggest the notion (24, 28) that the fusion pore of physiological exocytosis is formed by membrane lipids whose lateral diffusion is not prevented by immobile proteins (23). If the initial pore is formed from proteinaceous channels (3, 10), such channels would have to be disassembled before expansion of the pore to a diameter of 1.4 nm so as not to prevent lipid diffusion. Thus, the role of fusogenic proteins is more likely to be to control the proximity of two biological membranes and thereby to facilitate formation of the lipid nanopore. Such proteins may prevent lipid flux along the outer wall of the fusion pore, as described for hemagglutinin-induced cell fusion (29); the outer wall of the fusion pore might thus be proteinaceous to some extent. Given that insulin secretion is mediated by an expanding lipidic pore, abnormal lipid metabolism (14) in individuals with non-insulin-dependent (type 2) diabetes mellitus may impair secretion at the level of lipidic pore structure. Our approach provides a means of further elucidating the molecular control of fusion pore structures during physiological exocytosis.

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30. We thank H. Gaisano for critical reading of the manuscript; H. Noguchi for helpful discussion; and R. Ijuin, T. Kise, and N. Takahashi for technical assistance. This work was supported by Grants-in-Aid from the Japanese Ministry of Education, Culture, Sports, Science and Technology and the Japan Society for the Promotion of Science; by Research grants from the Human Frontier Science Program Organization, Uehara Memorial Foundation (H.K.), and Japan Diabetes Foundation (N.T.); and by PRESTO of the Japan Science and Technology Corporation (JST) (T.N.).

Supporting Online Material

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Materials and Methods

Supporting Text

Figs. S1 and S2

Movies S1 and S2

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Requirement for Caspase-2 in Stress-Induced Apoptosis Before Mitochondrial Permeabilization

Patrice Lassus, Ximena Opitz-Araya, Yuri Lazebnik*

A current view is that cytotoxic stress, such as DNA damage, induces apoptosis by regulating the permeability of mitochondria. Mitochondria sequester several proteins that, if released, kill by activating caspases, the proteases that disassemble the cell. Cytokines activate caspases in a different way, by assembling receptor complexes that activate caspases directly; in this case, the subsequent mitochondrial permeabilization accelerates cell disassembly by amplifying caspase activity. We found that cytotoxic stress causes activation of caspase-2, and that this caspase is required for the permeabilization of mitochondria. Therefore, we argue that cytokine-induced and stress-induced apoptosis act through conceptually similar pathways in which mitochondria are amplifiers of caspase activity rather than initiators of caspase activation.

Apoptosis is executed by caspases, a family of proteases that disassemble a cell (1, 2). The pathways leading to caspase activation vary with the cytotoxic stimulus. The stimuli that are collectively referred to as cytotoxic stress, such as DNA damage, activate caspases by initiating signaling pathways that converge on the Bcl-2 family of proteins (3). A balance between members of this family is thought to determine whether mitochondria remain intact or become permeabilized and release proteins that promote cell death (4). One of these released proteins is cytochrome c, which, in a complex with the cytoplasmic protein Apaf-1, activates caspase-9. Caspase-9, in turn, activates caspase-3, the protease that cleaves the majority of caspase substrates during apoptosis. Two other proteases, Smac (also called Diablo) and Htr2A (Omi), accelerate caspase activation and increase caspase activity by inactivating caspase inhibitors. Mitochondria also release apoptosis-inducing factor (AIF) and endonuclease G, which appear to kill independently of caspases. Therefore, mitochondria are thought to be a central regula-

tory element in stress-induced apoptosis (5).

Another way to activate caspases, used by cytokines, is to assemble receptor complexes that recruit caspase-8 or caspase-10, thereby inducing their autocatalytic processing (2). These caspases activate other caspases, including caspase-3, either directly (by proteolytic processing) or indirectly by cleaving Bid, a Bcl-2 family member. A proteolytic fragment of Bid permeabilizes mitochondria, thereby accelerating cell disassembly as described above. Hence, mitochondria in this pathway function as "amplifiers" of the caspase activity rather than as central regulators of apoptosis.

This model of apoptosis was consistent with studies of oncogene-dependent apoptosis, a phenomenon that may provide clues about how to kill cancer cells selectively (6). By comparing normal human fibroblasts (IMR90) with fibroblasts transformed with the adenoviral oncogene *E1A* (IMR90E1A), we found that this oncogene sensitizes cells to chemotherapeutic drugs by facilitating the activation of caspase-9 (7). *E1A* appears to achieve this effect by promoting the activation of Bax, a proapoptotic Bcl-2 protein that can permeabilize mitochondria, and by repressing a still-unidentified inhibitor of this permeabilization (8). These observations were in agreement with a model in which Bcl-2 proteins control caspase activation by regulating mito-

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chondrial permeability. Contrary to this view, we report that mitochondrial permeability is controlled by a caspase that is activated earlier in the pathway that links DNA damage and cell disassembly.

We used small interfering RNA (siRNA) to silence the expression of various proteins by RNA interference (RNAi) in IMR90E1A cells (9). A siRNA to caspase-2, a caspase that has been implicated in apoptosis but whose exact function and regulation remain unknown (10–14), efficiently and specifically silenced caspase-2 expression (Fig. 1A). Several other siRNAs (15) had no effect on the expression of caspase-2. Only the siRNA to Apaf-1 repressed the expression of Apaf-1, and none of the siRNAs used affected the expression of caspase-9 (Fig. 1A). The siRNA to Apaf-1 prevented apoptosis induced by all three DNA-damaging agents [etoposide, cisplatin, and ultraviolet (UV) light] that we used (Fig. 1B). To our surprise, however, inhibition of the expression of caspase-2 and of Apaf-1 prevented apoptosis with equal efficiency. This effect could not be attributed to manipulations required to introduce siRNAs into cells, because the siRNA to caspase-1 had no effect on apoptosis (Fig. 1B). Therefore, we conclude that either caspase-2 is required for apoptosis, or the caspase-2 siRNA interfered with the expression of other proteins.

To test whether apoptosis indeed required caspase-2, we attempted to restore sensitivity to cytotoxic agents by expressing caspase-2 ectopically. To prevent the destruction of the ectopic caspase-2 mRNA by the caspase-2 siRNA, we introduced two silent mutations into the region of the caspase-2 cDNA that is complementary to the siRNA (16). Consistent with the reported high specificity of siRNA (17), the expression of both the endogenous and ectopic caspase-2 from the wild-type cDNA was efficiently silenced, whereas the expression of caspase-2 from the mutated cDNA (caspase-2si) was not (Fig. 2A). Expression of caspase-2si restored the sensitivity of cells to etoposide (Fig. 2B), indicating that the effect of the siRNA could be explained by repression of caspase-2. Expression of catalytically inactive caspase-2si (in which catalytic cysteine was mutated to serine) (16) did not rescue the effect of the siRNA; this result indicates that the proteolytic activity of caspase-2 is required to mediate apoptosis (Fig. 3). The absence of caspase-2 did not affect the rate of apoptosis induced by tumor necrosis factor- α (fig. S1), which indicates that caspase-2 is not required for receptor-mediated apoptosis.

When cells lacking caspase-2 were exposed to etoposide, cytochrome c remained in mitochondria (Fig. 4A) (fig. S3), as did Smac (Fig. 4B) (fig. S3). Both proteins were released in the absence of Apaf-1, which indicates that experimental manipulations required to introduce siRNA into cells do not prevent mitochondria permeabilization. Caspase-2 was proteolytically processed even if processing of caspases 9, 3,

and 7 was prevented by the siRNA to Apaf-1; this finding suggests that caspase-2 is activated before or independently of the other three caspases (fig. S2). Hence, caspase-2 is required

to permeabilize mitochondria in these cells, a conclusion consistent with an observation that this caspase can permeabilize mitochondria in a cell-free system (18).

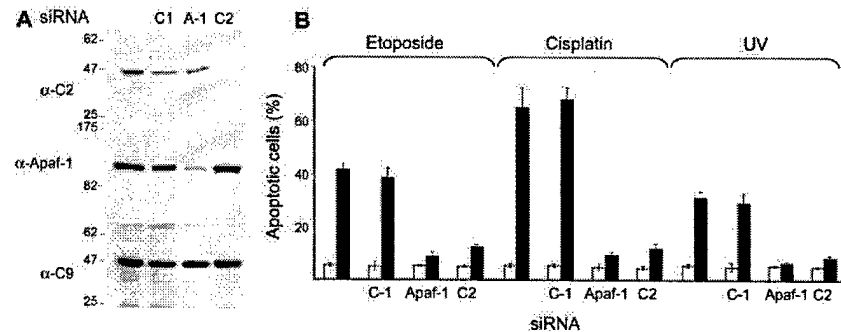


Fig. 1. Requirement of caspase-2 for apoptosis induced by several cytotoxic agents. (A) IMR90E1A cells were transfected with siRNA to Apaf-1 (A-1), caspase-2 (C2), or caspase-1 (C1) (as a control for the effect of transfection) or were left untransfected (16). After culturing cells for 2 days, a portion of the cells was used to determine amounts of caspase-2 and Apaf-1 by immunoblotting (16). Molecular mass markers (in kD) are shown. (B) The remaining cells were treated with the indicated cytotoxic agents (solid bars) or left untreated (open bars). The final concentration of the drugs in the medium was 50 μ M for etoposide and 20 μ M for cisplatin. UV was used at 10 mJ/cm². After 18 hours of treatment, both adherent and floating cells were collected, fixed with 4% paraformaldehyde, and stained with 4',6'-diamidino-2-phenylindole (DAPI) to reveal chromatin structure. Cells with condensed chromatin were scored as apoptotic.

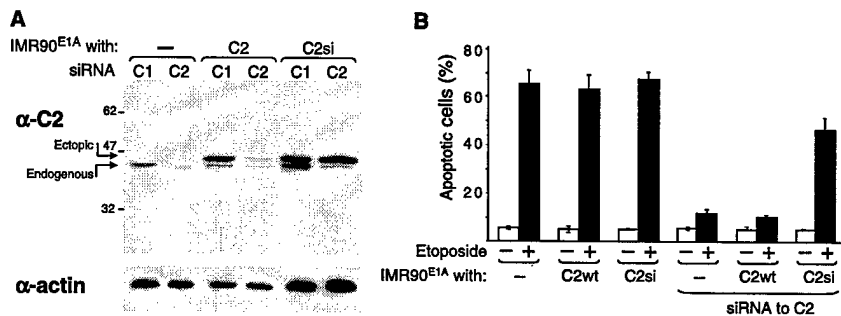


Fig. 2. Restored sensitivity of cells to apoptosis after ectopic expression of caspase-2. (A) cDNA encoding caspase-2 (C2) or caspase-2 in which two silent mutations prevented interaction between siRNA and the caspase-2 mRNA (C2si) (16) were ectopically expressed in IMR90E1A cells by retroviral transduction (16). The cell lines were transfected with siRNA to caspase-2 or caspase-1 or left untransfected. Two days later, the amount of caspase-2 in the cell lines was determined by immunoblotting (16). The ectopic protein contains a Myc epitope tag that allows distinction of endogenous and ectopic caspase-2 by a difference in electrophoretic mobility. The blot was probed with an antibody to β -actin to indicate the relative amount of total protein applied in each lane (16). (B) The cells transfected with siRNA as described in (A) were treated with 50 μ M etoposide or left untreated, and the rate of apoptosis was determined (Fig. 1).

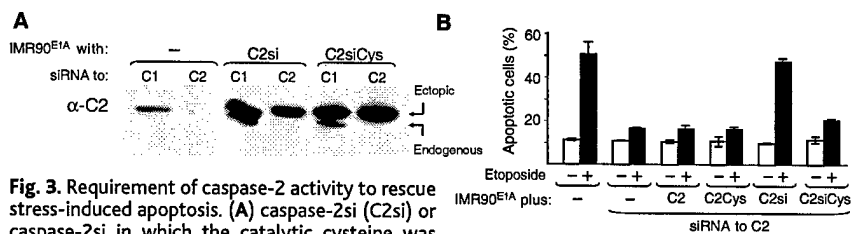


Fig. 3. Requirement of caspase-2 activity to rescue stress-induced apoptosis. (A) caspase-2si (C2si) or caspase-2si in which the catalytic cysteine was mutated into a serine (C2siCys) (16) were expressed in IMR90E1A cells by retroviral transduction (16). The cell lines were transfected with siRNA to caspase-2 or to caspase-1 (16), and expression of endogenous and ectopic caspase-2 was determined by immunoblotting. (B) The cells transfected with siRNA to caspase-2 as described in (A) or left untransfected were treated with 50 μ M etoposide for 18 hours or left untreated, and were then scored for apoptosis (Fig. 1).

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The earliest detectable change in the apoptotic machinery after DNA damage may be the translocation of the cytoplasmic Bcl-2 family member Bax to mitochondria (19). Etoposide induced Bax translocation in cells transfected with the siRNA to Apaf-1. However, Bax remained in the cytoplasm in cells transfected with the siRNA to caspase-2 (Fig. 4C) (fig. S3). Thus, caspase-2 is required to translocate Bax to mitochondria in this experimental system.

Hence, as previously suggested (20), our results imply that stress-induced apoptosis can be executed in a pathway that is conceptually similar to that of cytokine-induced apoptosis, in that each pathway begins with the activation of

a caspase that uses mitochondria to amplify the total caspase activity in the cell.

We expanded our study to five human tumor cell lines that can be efficiently transfected, are sensitive to cytotoxic agents, and are widely used in cancer research. In the lung adenocarcinoma A549 and the osteosarcoma U2OS, caspase-2 and Apaf-1 were required to produce morphological features of apoptosis (Fig. 5, A and B), and, in both cell lines, caspase-2 was required for cytochrome c release (Fig. 5C). In contrast, breast cancer cell line MCF-7 did not require caspase-2 for release of cytochrome c, perhaps because cytochrome c release is caspase-independent in these cells or because other caspases are involved (15). The results

obtained with cervix adenocarcinoma HeLa and colorectal carcinoma HCT-116 cells were inconclusive because the viability of these cells is poor when transfected with siRNA.

Our findings have several implications (16). Although strong evidence indicates that a failure of apoptosis contributes to cancer progression in experimental systems, the evidence is much weaker for such a relationship in human cancers (21). Evidence for the latter has been primarily gathered by correlating tumor properties with deficiencies in the apoptotic machinery. Our findings imply that a critical part of a major apoptotic pathway is yet to be considered by such studies. Indeed, the survival of cancer cells might be enhanced by any changes that prevent caspase-2 activation. How this activation is regulated is unknown, which indicates that even the basic pathways of apoptosis are not yet sufficiently understood to allow the efficient modulation of apoptosis to a therapeutic end. This study also highlights the ability of RNAi to yield results in cancer biology studies that do not depend solely on observations made with genetically modified mice.

Fig. 4. Requirement of caspase-2 for release of cytochrome c and Smac from mitochondria, and for translocation of Bax from the cytoplasm to mitochondria. IMR90E1A cells were transfected with siRNA to either Apaf-1 or caspase-2. After 2 days, the cells were either treated with 50 μ M etoposide or left untreated; the cells were fixed 18 hours after treatment, and cytochrome c, Smac, and Bax were visualized by immunofluorescence (16) (figs. S4 to S6). The fraction of cells in which cytochrome c or Smac was released from mitochondria (A and B) or Bax translocated from the cytoplasm to mitochondria (C) was determined by counting 400 to 500 cells for each cell population. The cells were counterstained with DAPI to visualize the nuclei.

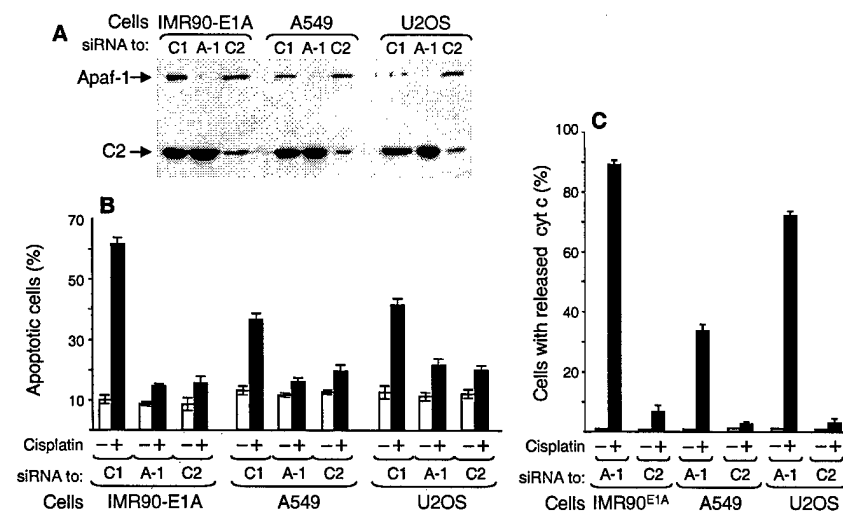
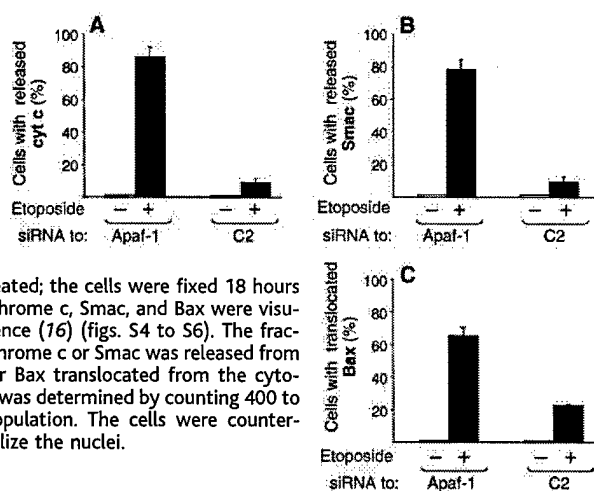


Fig. 5. Requirement of caspase-2 for apoptosis and cytochrome c release in human tumor cell lines. (A) The indicated cell lines were treated with siRNAs to caspase-1, Apaf-1, or caspase-2 (16), and expression of Apaf-1 and caspase-2 was determined by immunoblotting with antibodies to these proteins (16). The blot shown was exposed after incubation with both antibodies. (B) Cells transfected with siRNA to caspase-1, Apaf-1, or caspase-2 were treated with cisplatin (40 μ M) for 20 hours, then collected and scored for apoptosis by observing chromatin condensation (Fig. 1). (C) Cytochrome c release in the cells treated as described in (B) was visualized by immunofluorescence, and the fraction of cells with released cytochrome c was determined by counting about 500 cells.

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Supporting Online Material

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Figs. S1 to S3
References

4 June 2002; accepted 3 July 2002

Confirming Specificity of RNAi in Mammalian Cells

Patrice Lassus,* Joe Rodriguez,* and Yuri Lazebnik*

(Published 27 August 2002)

INTRODUCTION

MATERIALS

- siRNAs
- Cell Culture
- Retroviral Transduction
- Transfection
- Mutagenesis

EQUIPMENT

RECIPES

INSTRUCTIONS

- Gene Silencing Using siRNA
- Rescue of RNAi with a cDNA Containing Silent Mutations

RELATED TECHNIQUES

- Rescue by Targeting the 3' UTR

NOTES AND REMARKS

REFERENCES

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Abstract

RNA interference (RNAi) is a process of sequence-specific gene silencing. Recent advances in the understanding of RNAi have provided practical tools to silence gene expression in mammalian cells, opening new possibilities for studying the functions of genes and proteins. It is important to ensure that an observed effect of RNAi is due to silencing of the intended target. Indeed, it is possible that an siRNA may silence more than one messenger RNA that is homologous in the region complementary to the siRNA. Considering that we know little about how RNAi works in mammalian cells, other artifacts may be yet to be recognized. Thus, we suggest approaches to rescue the effect of RNAi by ectopically expressing the protein of interest. These approaches involve introducing silent mutations into the complementary DNA of the protein and targeting RNAi to the untranslated regions of the gene.

Introduction

Preventing expression of a protein is an effective way of learning what this protein actually does or does not do. Until recently, this approach required a substantial amount of effort and was largely limited to animal models. The advent of RNA interference (RNAi) allows one to silence gene expression in various types of cells, including human, which has opened new possibilities that biologists have only dreamed of. We refer the reader to a recent review (1) for a detailed description of RNAi technology and its underlying mechanisms. From the point of view of the practitioner, RNAi is a phenomenon that allows one to destroy an mRNA by introducing into a cell a double-stranded RNA that is the cognate of the target gene. Currently, the double-stranded RNA can be introduced by transfection as a short synthetic or in vitro transcribed RNA duplex (siRNA) (2), or expressed from an appropriate vector either as a hairpin RNA or as two separate strands (3, 4). Because the field is relatively new and is developing rapidly, it is likely that, by the time you read this protocol, understanding of RNAi and the number of tools available for silencing genes will have expanded.

Whatever the tool, you would want to confirm that the observed effect of RNAi is indeed due to silencing of the intended target. A common way to test whether an effect is due to a deleted gene is to express the product of this gene ectopically. However, RNAi will silence expression of both endogenous and ectopic messenger RNA (mRNA). We suggest two ways to overcome this problem (Fig. 1). One method is to use a complementary DNA (cDNA) that has silent mutations in the region that is targeted by RNAi (Fig. 1A). These mutations prevent interaction of the RNAi machinery with the ectopic mRNA, but do not destroy the endogenous mRNA (5). The second approach (Fig. 1B) is to target RNAi to an untranslated region (UTR) of the gene of interest and to rescue the effect by expressing a cDNA that encodes the wild-type coding sequence but does not contain the native untranslated region (UTR).

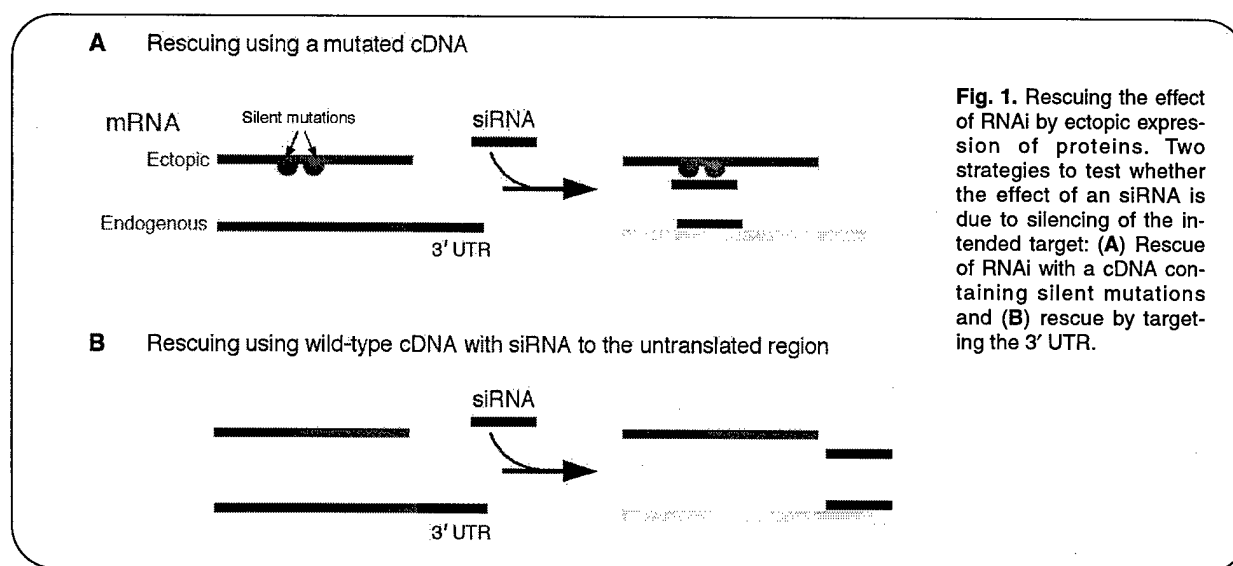


Fig. 1. Rescuing the effect of RNAi by ectopic expression of proteins. Two strategies to test whether the effect of an siRNA is due to silencing of the intended target: (A) Rescue of RNAi with a cDNA containing silent mutations and (B) rescue by targeting the 3' UTR.

Materials

siRNAs

Control siRNAs; for example, siRNA complementary to lamin A [Dharmacon (<http://www.dharmacon.com/>)]

siRNAs complementary to the target gene (Dharmacon)

Cell Culture

3-ml syringes

Six-well tissue culture dishes (Falcon)

10-cm tissue culture dishes (Falcon)

Dulbecco's Modified Eagle's Medium (DMEM) with L-glutamine [Gibco (<http://www.lifetech.com/>)]

Fetal bovine serum (FBS) (Gibco)

IMR90-E1A, which are human primary fibroblasts IMR90 [American Type Culture Collection (ATCC) #CCL-186] transformed with the adenoviral oncogene E1A (6)

Trypsin-EDTA (Gibco)

Retroviral Transduction

0.45- μ m syringe filters (Millipore)

N,N-bis [2-hydroxyethyl]-2-aminoethane-sulfonic acid (BES) (Sigma-Aldrich)

CaCl₂

Deionized H₂O

Hepes

KCl

KH₂PO₄

MaRX vectors, such as pMaRX-hygro (provides resistance to hygromycin) or pMaRX-puro (provides resistance to puromycin) (7). The vectors are available from G. Hannon (hannon@cshl.org).

NaCl

Na₂HPO₄

Packaging cells [we use LinX, which are also available from G. Hannon (hannon@cshl.org)]

Pasteur pipettes

Polybrene [Sigma-Aldrich (<http://www.sigma-aldrich.com/>)]

Siliconized 1.7-ml microcentrifuge tubes

Transducing cells (such as IMR90-E1A cells or other cell lines)

Transfection

Oligofectamine transfection reagent [Invitrogen (<http://www.lifetech.com/>)] or Fugene [Roche (<http://www.roche-applied-science.com/>)]

OptiMEM (Gibco)

Mutagenesis

QuikChange Site-Directed Mutagenesis Kit (Stratagene) (<http://www.stratagene.com/manuals/200518.pdf>)

Equipment

Automatic pipetter (such as PipetAid)
Microcentrifuge
Multiblock heater
PCR machine
Tabletop centrifuge with a rotor for six-well tissue culture plates
Tissue culture hood
Water bath

Recipes

Recipe 1: PBS

NaCl	8 g
KCl	0.2 g
Na ₂ HPO ₄	1.12 g
KH ₂ PO ₄	0.24 g

Dissolve in 800 ml of dH₂O. Adjust pH to 7.3 with concentrated HCl if needed and adjust the volume to 1 liter. Autoclave the solution.

Recipe 2: Growth Medium

Under sterile conditions, add FBS to DMEM to 10% v/v. Do not add antibiotics.

Recipe 3: CaCl₂ Solution

CaCl ₂	2.5 M
Hepes	10 mM

Adjust the pH to 5.5 with concentrated HCl.

Recipe 4: 2× BBS

BES	50 mM
NaCl	280 mM
Na ₂ HPO ₄	1.5 M

Adjust the pH to 6.95 with 10 M NaOH.

Recipe 5: Polybrene Solution

Polybrene 8 mg/ml in deionized H₂O

Instructions

We will describe how to rescue the effect of RNAi using silencing by synthetic siRNA as an example. However, the principles of the rescue are likely to be applicable to other RNAi techniques. The instructions explain in detail how to use siRNA in mammalian cells and describe how to test whether the observed effect of siRNA is due to silencing of the intended target.

Gene Silencing Using siRNA

Application of siRNA in mammalian cells involves the following steps: (i) choosing the siRNA sequence, (ii) obtaining the siRNA from a supplier, (iii) transfecting the siRNA, (iv) testing efficiency of gene silencing, and (v) determining whether the silencing causes any changes in the experimental system.

Selection of siRNA sequences

We follow the guide (8) provided by T. Tuschl (<http://www.mpibpc.gwdg.de/abteilungen/100/105/index.html>), who pioneered the siRNA approach. A general rule is that the sequence should be AA(19N)TT (where N is any nucleotide) with a GC content of 30 to 70%. A computer program developed by Lin (9) in our laboratory uses this rule to select potential siRNA sequences and determine whether the selected sequences match cDNA sequences other than those of the intended target. In addition, it is important to consider that a guanine base in siRNA and the target RNA may pair not only with a cytosine, but also with a uracil. In principle, both coding and UTR of the mRNA can be targeted. However, targeting siRNA to UTR is not recommended, because regulatory protein complexes that bind to these regions may interfere with the binding of the RNAi machinery. Despite this potential problem, we found that five out of six siRNA that target the 3' UTR of six genes are very efficient, and we suggest using this approach if specificity of silencing is confirmed (Fig. 2).

Although the available rules for siRNA selection are a reliable general guide, they do not guarantee that each of the selected siRNAs will work. Therefore, you will need to decide how many siRNAs to order, a choice that will be determined by your budget and the time that you can wait for the results. If you are on a tight budget, ordering siRNA one at a time may be an acceptable approach, because there is about a 50% chance that the first siRNA you order will work well. If money is not an issue, we suggest ordering four siRNAs, two for the coding sequence and two for the 3' UTR. The companies that provide synthetic siRNA and descriptions of their products can be found at the Tuschl laboratory Web site (<http://www.mpibpc.gwdg.de/abteilungen/100/105/index.html>). We have used Dharmacon, because we have found their service reliable.

We also suggest using two siRNAs as controls: one that has no effect on the cells being tested (for example, an siRNA targeting a gene that is not expressed in these cells), and another that has a known and an easily tested effect, such as the siRNA to lamin A. The siRNA that is targeted to lamin A is available commercially.

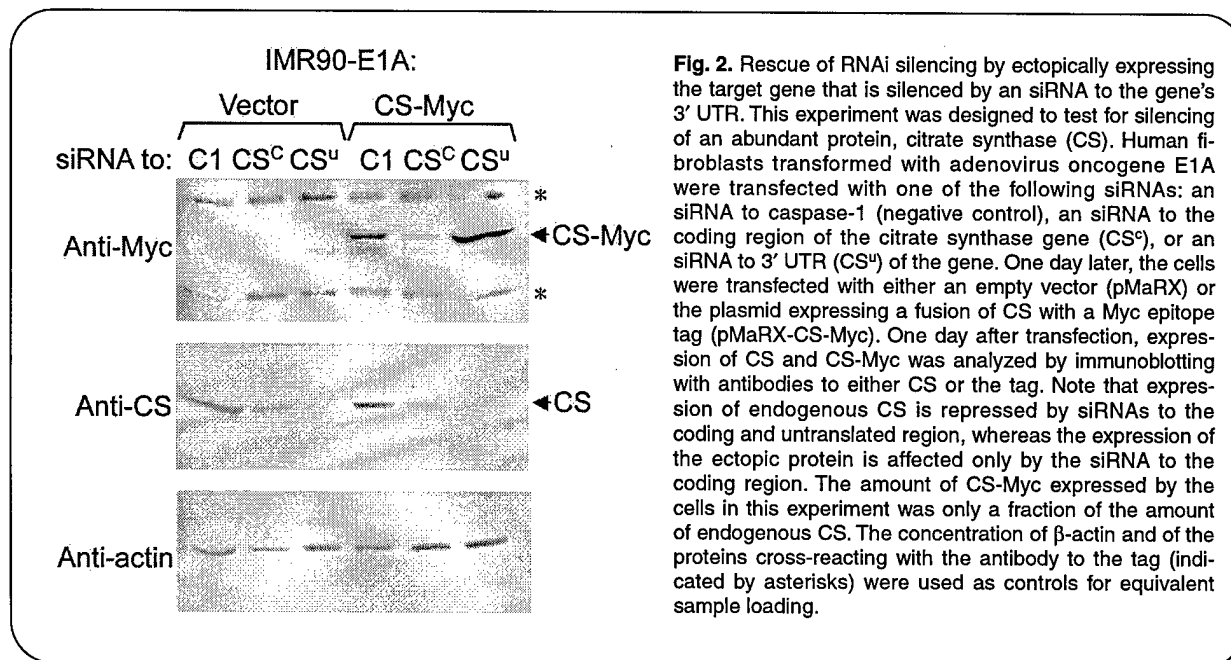


Fig. 2. Rescue of RNAi silencing by ectopically expressing the target gene that is silenced by an siRNA to the gene's 3' UTR. This experiment was designed to test for silencing of an abundant protein, citrate synthase (CS). Human fibroblasts transformed with adenovirus oncogene E1A were transfected with one of the following siRNAs: an siRNA to caspase-1 (negative control), an siRNA to the coding region of the citrate synthase gene (CS^C), or an siRNA to 3' UTR (CS^U) of the gene. One day later, the cells were transfected with either an empty vector (pMaRX) or the plasmid expressing a fusion of CS with a Myc epitope tag (pMaRX-CS-Myc). One day after transfection, expression of CS and CS-Myc was analyzed by immunoblotting with antibodies to either CS or the tag. Note that expression of endogenous CS is repressed by siRNAs to the coding and untranslated region, whereas the expression of the ectopic protein is affected only by the siRNA to the coding region. The amount of CS-Myc expressed by the cells in this experiment was only a fraction of the amount of endogenous CS. The concentration of β-actin and of the proteins cross-reacting with the antibody to the tag (indicated by asterisks) were used as controls for equivalent sample loading.

Preparation of siRNA for transfection

The supplier we use, Dharmacon, provides three options for purchasing siRNA. The most expensive option provides RNA duplexes that are ready for transfection; with this option, the three steps below are unnecessary. However, we usually use the less expensive option B, which provides purified, lyophilized, single-stranded oligonucleotides that are ready to anneal according to the following steps.

1. Resuspend the oligonucleotides in the water provided by the supplier.
2. Anneal the oligonucleotides according to the manufacturer's instructions.
3. Store the resulting 20- μ M siRNA solutions in 50 μ l aliquots at -70°C .

Transfection of siRNA

Transfection of siRNA is the most critical factor in the success of gene silencing. Transfection conditions depend on the cells and the transfection reagents. The procedure that we describe below has been optimized for human fibroblasts, such as IMR90, transformed with the adenoviral oncogene E1A [(6) and the Oligofectamine transfection reagent]. We have found that this procedure is also effective with at least some other cell lines, such as HeLa, MCF7, U2-OS, and A549.

Note: All procedures are should be performed in a tissue culture hood at room temperature. PBS (Recipe 1) and Growth Medium (Recipe 2) should be warmed to 37°C before use.

1. Plate cells in 6-well plates such that the cells will be 25 to 30% confluent at the time of transfection.

Note: Cell density is very important. Not enough cells per plate will result in high background toxicity; too many cells will lower transfection efficiency. The right cell density will have to be determined empirically, but a good starting point is to initiate cultures with about 10^5 cells per well.

2. Incubate cells overnight in an incubator set at 37°C and 3% CO_2 .
3. Set up a siliconized microcentrifuge tube for each 20- μ M siRNA (including controls). This will be Tube 1 for each siRNA. Gently mix together 10 μ l of the siRNA duplex with 200 μ l of OptiMEM.

Note: Mixing can be done by pipetting the solution or inverting the tube. Do not vortex to mix.

4. In a second siliconized microcentrifuge tube (Tube 2) for each siRNA (including controls), gently mix 10 μ l of Oligofectamine with 50 μ l of OptiMEM.
5. Allow the samples to sit for 5 min at room temperature.
6. For each siRNA, combine the contents of Tube 1 and Tube 2, and mix gently by pipetting or inverting. This is the transfection mixture.

Note: Do not vortex to mix.

7. Allow the transfection mixtures to sit 20 to 25 min at room temperature.
8. Meanwhile, rinse cells with 2 ml of PBS (Recipe 1), and add 2 ml of Growth Medium (Recipe 2) per well.
9. Add the transfection mixture dropwise to the cells while gently agitating the plate.
10. Incubate the cells overnight in an incubator set at 37°C and 3% CO_2 with a humidified atmosphere.

Note: If the transfection is toxic to the cells, the cells can be rinsed with PBS (Recipe 1) after 6 hours and Growth Medium (Recipe 2) added.

11. Replace the transfection medium with 2 ml of Growth Medium (Recipe 2) per well.
12. Incubate the cells in an incubator set at 37°C and 3% CO_2 with a humidified atmosphere until the target protein has disappeared from the cells.

Note: It may take several days for the siRNA to be effective, depending on the efficiency of siRNA and the stability of the protein. We found that 2 days after siRNA transfection was optimal to decrease the concentration of some proteins, whereas others required 4 days. The timing for each experimental system should be determined empirically.

Evaluating the efficiency of siRNA

If the functional product of the gene is a protein, two direct ways of measuring the effectiveness of siRNA are immunoblotting or immunofluorescence. If a specific antibody is not available, one possibility is to ectopically express the protein as a fusion with an epitope tag, such as hemagglutinin (HA) or Myc, and use a commercial tag-specific antibody to determine protein expression. This approach is based on a reasonable assumption that expression of the endogenous protein will be as efficiently silenced as that of the ectopic. If an epitope-tagged version of the protein is used to test for expression levels, then the sequence targeted by RNAi should also be part of the ectopically expressed mRNA.

Rescue of RNAi with a cDNA Containing Silent Mutations

This method uses ectopic expression of the target protein from a cDNA that contains silent mutations in the sequence complementary to the siRNA (Fig. 1A). siRNA should silence expression of the endogenous protein and the protein that is expressed from the native, but not from the mutated, cDNA. If this is the case, determine whether the phenotype being investigated is observed only in cells that do not express the protein. For an example, see our study that describes silencing of caspase-2 (5). This next section of the protocol describes how to mutate a cDNA and presents methods for either retroviral transduction or transfection of the wild-type and mutated cDNAs.

There are no rules for choosing the bases to mutate except that the mutations must be silent. Consistent with published reports (10), we found that two mutations were sufficient to prevent the destruction of the mRNA by the siRNA, although introducing more mutations would probably only help. The mutations are more efficient if placed together and as close as possible to the middle of the sequence complementary to the siRNA, a region that is required for siRNA silencing (10). The mutations can be introduced by any of several methods. We follow the protocol provided with the QuikChange Site-Directed Mutagenesis Kit by Stratagene, which gives consistently reliable results. To aid in the screening of mutants, it is useful to combine silent mutations with a restriction site. The Webcutter program (11) is helpful for this purpose.

Once you have generated a mutant cDNA, you can attempt to rescue the phenotype obtained with the siRNA by expressing the cDNA in cells. Two methods we describe in detail below are retroviral transduction and transient transfection. We prefer the former because it provides a population of cells rather than single clones, and it allows one to obtain a sufficient amount of cells within 2 weeks. Regardless of the approach you choose, you will need to confirm that siRNA results in silencing of expression of the endogenous, but not of the ectopic, gene. A convenient way of distinguishing the endogenously and ectopically expressed proteins is to express the ectopic protein as a fusion with an epitope tag. The tag may change the mobility of the protein in gel electrophoresis, which will separate the ectopic and endogenous proteins, and allow for detection of the ectopic protein independently of the endogenous (Fig. 2).

Retroviral transduction

The first step is to clone the mutated, as well as the native, cDNA into a retroviral vector containing a drug selection gene. Various systems for retroviral transduction are available. We often use the MaRX set of vectors (7), which has been consistently efficient with our experimental systems. We also use pBABE retroviral vectors (12). When planning the number of cells needed for retroviral transduction, consider that you will need cells that express the mutated or native cDNA, as well as cells that are not transfected by the retroviral vector ("mock-transduced") as a control for selection.

Remember that even a retrovirus deficient in proliferation should be handled with caution. You may not want to express your favorite protein in yourself. Follow exactly your institution's rules governing the use of retroviruses and use common sense.

Note: All solutions should be sterile.

1. Split the packaging line (LinX) into six-well plates so that they will be 70 to 80% confluent the next day.

Note: Plan to have enough packaging cells to include nontransfected controls in addition to those for transfection with the mutant and wild-type cDNA.

2. On the day of transfection, replace the media with 2 ml of fresh Growth Medium (Recipe 2) per well.
3. Return the cells to the incubator set at 37°C and 3% CO₂ for at least 1 hour.
4. For each well containing cells to be transfected, aliquot 6 µg of plasmid (mutated cDNA or wild-type cDNA in a pMaRX vector) into separate 1.7-ml microcentrifuge tubes, adjust the volume to 225 µl with deionized H₂O, and add 25 µl of CaCl₂ Solution (Recipe 3).
5. Add dropwise 250 µl of 2× BBS (Recipe 4) while bubbling air through the solution with a Pasteur pipette connected to a PipetAid.

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6. Add the resulting solution dropwise to the cells while swirling the plate.
7. Incubate at 37° and 3% CO₂ overnight in an incubator.
8. Replace the medium with 2 ml of fresh Growth Medium (Recipe 2) and incubate at 32° and 3% CO₂ for 60 hours (2.5 days).
9. On the day before transduction, split the cells to be transduced into a six-well plate such that they will be 50 to 60% confluent at the time of transduction. Use 2 ml of Growth Medium (Recipe 2) media per well.
10. Collect the medium containing retrovirus from the packaging line with a 3-ml syringe.
11. Attach a 0.45- μ m filter to the syringe and pass the retrovirus-containing medium through the filter onto cells to transduce. This will result in a volume of 4 ml of medium per well (2 ml of medium with the virus added to 2 ml of medium already in the well).
12. Add supernatant from untransfected packaging cells to one of the wells. These cells will be called "mock-transduced."
13. Add 4 μ l of 8 mg/ml Polybrene Solution (Recipe 5) to each well (final concentration of 8 μ g/ml).
14. Spin the plate in a tabletop centrifuge at 1700 rpm for 1 hour at room temperature.
15. Incubate at 32°C and 3% CO₂ overnight.
16. Change the medium and incubate at 37°C and 3% CO₂ for 2 days (or until confluent).
17. Transfer the cells from each well into a 10-cm tissue culture dish.
18. Incubate at 32°C and 3% CO₂ overnight.
19. Add the appropriate selection drug, such as puromycin or hygromycin, which will depend on the vector used.
Note: The appropriate drug concentration will depend on the cells used and must be determined empirically.
20. Maintain cells in this selection medium until all mock-transduced cells die.
Note: If selection requires longer than one week, the medium should be replaced with fresh selection medium every 4 days.
21. Test for protein expression by immunoblotting or immunofluorescence.
Note: We recommend preparing a stock of frozen cells from cells that are positive for protein expression before doing any experiments.
22. Transfect these cells with the siRNA and determine whether the mutated cDNA rescued the phenotype of interest.

Transient transfection

If the cell line being tested can be transfected with a high efficiency (more than 80%), or if the phenotype can be studied at the single cell level (for example, by immunofluorescence), then transient transfection may be suitable. An example of this approach is given in Fig. 2. The conditions resulting in high efficiency with low toxicity must be determined empirically by varying cell density and transfection reagents. We routinely use Fugene from Roche or Lipofectamine 2000 from Invitrogen and follow the instructions provided by the manufacturers. We recommend that the cDNA be transfected at least one day after transfection of the siRNA. The following typical procedure is performed at room temperature in a laminar flow hood to transfect cells plated in six-well tissue culture plates.

1. Label one 1.7-ml siliconized microcentrifuge tube for each plasmid.
2. Add 100 μ l of OptiMEM into each tube.
3. Add 5 μ l of Fugene and mix gently by pipetting.
4. Add 2 to 3 μ g of plasmid (mutated cDNA or wild-type cDNA) and mix gently by pipetting.
5. Wait 20 to 25 min.
6. Rinse the cells previously transfected with the siRNA with 2 ml of PBS.
7. Add 2 ml of fresh Growth Medium (Recipe 2) per well.
8. Add the transfection mixture dropwise to the cells while gently agitating the plate.
9. Incubate the cells at 37°C and 3% CO₂ overnight in an incubator.

Note: If the transfection is toxic to the cells, then the transfection medium can be replaced with Growth Medium after 4 to 8 hours.

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10. Replace the medium with 2 ml of Growth Medium (Recipe 2) per well.
11. Incubate the cells at 37°C and 3% CO₂ for 1 to 2 days before collecting the cells.
12. Test for protein expression by immunoblotting or immunofluorescence. If the protein is not detectable, optimize the transfection conditions or consider retroviral transduction.

Related Techniques

Rescue by Targeting the 3' UTR

If the siRNA is directed to the 3' UTR of the gene, the effect of the siRNA can be rescued by ectopically expressing the protein using the wild-type cDNA. You would need to make sure, however, that the expression plasmid does not contain the targeted sequence of the 3' UTR of the gene of interest. This rescue method is more practical if you have a collection of plasmids that express the gene and various mutants. Thus, an siRNA directed against the 3' UTR would allow the function of all of these mutants to be analyzed. The steps required to apply this approach are the same as those described above, except the cDNA of the protein being tested does not need to be mutated. An example of this approach is provided in Fig. 2.

Notes and Remarks

Applications of RNAi in mammalian cells are likely to expand in scope and variety in the near future, which may likely affect how particular steps of this protocol should be implemented. We would appreciate comments and suggestions.

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