Identification of a Network Involved in Thapsigargin-induced Apoptosis Using a Library of Small Interfering RNA Expression Vectors*

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We describe here the construction of a library of small interfering RNA expression vectors targeted to a few hundred apoptosis-related genes and the application of this library to an investigation of thapsigargin (TG)-induced apoptosis. Thapsigargin triggers endoplasmic reticulum stress, with subsequent apoptosis, but the molecular mechanisms underlying this process are incompletely understood. Using our library, we identified three anti-apoptotic genes, namely, NOXA, E2F1, and MAPK1, in addition to already characterized genes in the apoptotic pathway. In contrast to proposals by others, our data revealed (i) that TG-induced apoptosis is associated with Apaf1 in a caspase-3- and caspase-9-independent manner; (ii) that the E2F1-PUMA pathway might be involved; and (iii) that the ERK pathway, via MAP3K8 (mitogen-activated protein kinase kinase 8), is required for the induction by TG of apoptosis. Our study demonstrates clearly that unexpected and novel genes can be identified effectively by our method, and it provides evidence for the efficacy and utility of the comprehensive analysis of signaling networks and pathways using a library of small interfering RNA expression vectors.

Although the human genome has been sequenced, the functions of many genes remain unknown. Phenotypic analysis using gene silencing appears to be an effective strategy in efforts to understand gene functions, and exploitation of RNA interference (RNAi)* appears to be a novel and powerful approach to the silencing of specific genes (1). RNAi is an intrinsic and evolutionarily conserved phenomenon in plants and animals whereby double-stranded RNA induces the sequence-specific degradation of homologous RNA (2).

Genome-wide RNAi screening in Caenorhabditis elegans and Drosophila cells, using libraries of double-stranded RNAs, has been demonstrated to be extremely useful in efforts to identify genes that regulate various processes and has enhanced our understanding of various biological processes (3–6). However, two major problems are associated with such screening in mammalian cells. First, double-stranded RNA induces an antiviral response that can result in cell death (7). However, Tuschi and co-workers (8) demonstrated that 21- and 22-nucleotide small interfering RNAs (siRNAs) can induce RNAi in the absence of an antiviral response in cultured mammalian cells. Subsequently, various groups, including our own, have developed systems for vector-mediated specific RNAi in mammalian cells (9–16). The second problem is that the effectiveness of siRNA is strongly dependent on the target sites in target RNAs. To generate a high quality library, we developed an original algorithm that allows us to predict favorable target sites (17). Using this algorithm and our optimized siRNA expression system (17), we are now constructing an siRNA expression library that will encompass the complete array of transcripts from the human genome. In this study, for construction of our first siRNA library, we focused on an apoptotic pathway and generated a library targeted to apoptosis-related genes. We used this library to screen for genes involved in endoplasmic reticulum (ER) stress-dependent apoptosis induced by thapsigargin (TG), a plant-derived sesquiterpene lactone.

** Experimental Procedures

Culture and Transfection of Cells—HCT116 cells were cultured in McCoy’s 5A medium, supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Transfections of siRNA expression vectors were performed using Lipofectamine™ 2000 (Invitrogen) according to the manufacturer’s protocol.

Construction of a Library for Expression of U6-driven siRNA—We prepared siRNA vectors using the vector pGENE PURhU6 (17), which contains a human U6 promoter, a puromycin resistance gene, and two BspMI sites, which are used as sites for cloning a short hairpin sequence. For construction of siRNA vectors, we synthesized oligonucleotides with hairpin, terminator, and overhanging sequences. We annealed these fragments and inserted them into the BspMI sites of pGENE PURhU6. We used the sequences that we inserted immediately downstream of each U6 promoter on the results of application of our algorithm (17). Each vector was designed to express a short hairpin RNA in transfected cells.

Western Blotting Analysis—Preparation of cell lysates, Western blot-
Identification of ER Stress by RNAi Library

RESULTS

Construction of the siRNA Expression Vector Library—Prior to the construction of the siRNA expression library, we optimized our siRNA expression system. We constructed three types of siRNA vector system, namely, tandem-type, dual promoter-type, and hairpin-type, and compared the activities of the siRNAs. We determined that the hairpin-type system had the highest suppressive activity for low copy number plasmids. However, we observed a high rate of mutation in the stem-loop region when plasmids were used to transform *Escherichia coli*. This phenomenon was a serious problem with respect to the construction of a reliable library of siRNA vectors. We found that introduction of multiple C to T (or A to G) mutations into the sequence that connected sense and antisense sequences and other parameters that might affect the activity of the siRNA. Furthermore, we developed an algorithm for selection of effective target sequences (17). Together, the optimization of the siRNA expression system and the development of the algorithm enabled us to construct a large scale and high quality library of siRNA vectors.

Thapsigargin-induced Caspase-dependant Apoptosis—We focused on the ER stress-induced apoptotic pathway as the target of our initial siRNA expression library. We chose 241 apoptosis-related genes, including genes for kinases, caspases, transcription factors, and other already characterized genes that are known to be associated with various apoptotic pathways. To take off-target effects into account (19), we constructed siRNA vectors directed at two target sites within each gene. Although there are many reports of the involvement of a number of specific genes in ER stress-induced apoptosis, the signal transduction pathway remains to be fully defined. Therefore, to clarify the details of the ER stress-induced apoptotic pathway, we screened the siRNA expression library for siRNA that targeted genes that might be involved in TG-induced apoptosis. First, we confirmed that cell death caused by TG is, in fact, apoptosis under our experimental conditions. TG clearly induced apoptotic cell death in human colon carcinoma HCT116 cells, as demonstrated by fluorescence-activated cell sorter analysis, during which we observed a characteristic sub-G1 peak, indicative of DNA fragmentation and a hallmark of apoptosis, in the case of TG-treated cells (Fig. 1A). Moreover, cleavage of anti-poly(ADP-ribose) polymerase, another hallmark of apoptosis, occurred 24 h after the start of treatment of cells with TG (Fig. 1B).

Incubation of cells with both TG and a pan-caspase inhibitor, z-VAD-fmk or the caspase-8 inhibitor z-IETD-fmk significantly blocked the appearance of the sub-G1 peak, indicating that the apoptotic process was caspase-dependent and, specifically, caspase-8-dependent. We also examined the possible role of a death receptor (DR5), which is involved in the induction of apoptosis via the recruitment and the cleavage of caspase-8, in TG-induced apoptosis in HCT116 cells. Recent studies have implicated the death receptor (DR5) pathway in TG-induced apoptosis in several lines of cancer cells (20). TG induced the expression of the DR5 protein, which became apparent 12 h after the onset of treatment, and “knockdown” experiments using an siRNA vector targeted to DR5 mRNA revealed that the DR5 apoptotic pathway was blocked by this vector (see below). Collectively, our results suggested that TG-induced apoptosis involved the DR5-mediated activation of caspase-8.

Confirmation of the RNAi Effect on Endogenous Genes—Prior to this study, we developed an algorithm for the identification of target sites using siRNA data sets obtained with reporter genes, such as genes for luciferases and green fluorescent protein (17). In this study, we examined whether or not the algorithm is applicable to endogenous genes. We constructed siRNA vectors directed against genes for caspases 3, 7, 8, and 9 and DR5, all of which might be involved in TG-induced apoptosis. As noted above, we used two different target sites per gene in these experiments. As shown in Fig. 2, Western blotting analysis indicated that the level of each respective protein was markedly reduced in cells that had been transfected with the siRNA vector targeted to the corresponding gene, whereas the control vector, targeted to the gene for the unrelated luciferase.
from Renilla (Rluc) had no effect. The levels of actin were unchanged, indicating that the effects of the siRNA vectors were specific to each respective gene (Fig. 2, A–E). These results suggested that our algorithm had allowed us to select appropriate sequences in endogenous genes (17). We also investigated the effects of siRNA vectors targeted to other genes by Western blotting. We confirmed that 70–80% of our siRNA vectors targeted to endogenous genes were able to silence these target genes very effectively.

Screening Strategy—We set the conditions for screening our siRNA expression library for the siRNAs that targeted genes involved in TG-induced apoptosis using an siRNA vector targeted to caspase-8 mRNA as a positive control. First, we investigated the duration of the effects of RNAi at the protein level after transfection of cells with this siRNA vector. To remove nontransfected cells, we treated cells with 2 μg/ml puromycin for 36–48 h and then analyzed levels of caspase-8. Reductions in levels of caspase-8 were sustained for at least 72–120 h after transfection, and inhibitory effects on apoptosis were observed over the same time range (Fig. 2F and data not shown). Under similar conditions, we used the siRNA expression library to screen for apoptosis-related genes. Thus, 36 h after transfection, we selected transfected cells by exposing cells to 2 μg/ml puromycin for 36 h. Then same number of cells were transferred in triplicate to a 96-well plate. After further 12 h, we treated with 1 μM TG for another 48 h. Then we fixed the cells and stained them with crystal violet. Negative control cells that had been transfected with siRNA vectors targeting to Rluc and other unrelated transgenes were all dead by the treatment of TG (Fig. 2F, right lane). Under this condition, siRNA expression vector library was screened, and we selected siRNA vectors that made cells survive after treatment with TG. We identified the siRNA vectors specific for several genes that suppressed TG-induced apoptosis (Table I and Fig. 2H). Targeted genes were divided into three classes according to the extent of cell survival.

Caspases—The strong inhibitory effect of the siRNA vector for the gene directed against caspase-8 indicated that this enzyme is a key molecule in TG-induced apoptosis. Cells transfected with siRNA vectors directed against DR5 mRNA resisted TG-induced apoptosis, but their survival was poorer than that of cells that had been transfected with siRNA vectors against the gene for caspase-8. In terms of protein levels, DR5-specific siRNA vectors almost completely suppressed the expression of DR5 (Fig. 2D), suggesting the possible existence of a DR5-independent pathway(s) for activation of caspase-8 that involves other receptors (such as FAS and tumor necrosis factor α receptors) or, perhaps, of a receptor-independent caspase activation pathway(s).

The suppression caused by siRNA vectors directed against Apaf1, VDAC, and Bid indicated that the TG-induced apoptotic pathway also involved mitochondria. By contrast, the siRNA

![Fig. 2. Confirmation of the RNAi effect on endogenous genes.](http://www.jbc.org/) HCT116 cells were transfected with siRNA vectors directed against two target sites in the mRNA for caspase-3 (A), caspase-7 (B), caspase-9 (C), DR5 (D), caspase-8 (E), and Renilla luciferase (Rluc), as a control. F–H, surviving cells after treatment with TG and the indicated siRNA expression vector for 48 h. The living cells were stained with crystal violet. The results are representative of three independent experiments.
vectors targeted to genes for caspase-9 and caspase-3 failed to suppress TG-induced apoptosis (Fig. 2G) despite the almost complete suppression of the expression of these two genes (Fig. 2, A and C). However, siRNA vectors directed against genes for caspase-3 and caspase-9 did block apoptosis that was induced by stimuli other than TG (e.g. double-stranded RNA; data not shown). Although it has been proposed that both caspase-9 and Apaf1 are necessary for the activation of downstream caspasas, Ho et al. (21) recently reported the presence of a caspase-9-dependent and Apaf1-independent apoptotic pathway in myoblast. Our data indicate that TG-induced apoptosis is dependent on Apaf1 but not on caspase-9 or on caspase-3, leading to the first identification, to our knowledge, of components of an apoptotic pathway downstream of mitochondria.

**Bel-2 and BH3-only Protein Families**—The siRNA vectors targeted against the Bad, PUMA, and NOXA genes for the members of the Bel-2 homology domain 3 family (the BH3-only protein family) inhibited TG-induced apoptosis. When pro-apoptotic members of the Bel-2 family, such as Bax and Bak, are activated, they increase the permeability of the outer mitochondrial membrane and induce the release of pro-apoptotic proteins from the mitochondria. In normal cells, anti-apoptotic members of the Bel-2 family, such as Bcl-2 and Bcl-xL, inhibit the mitochondrial membrane and induce the release of pro-apoptotic proteins from the mitochondria. When levels of cytoplasmic Ca2+ ions rise, after treatment of cells with TG, Bad is activated and translocated to mitochondria (22). Thus, Bad might be a candidate for a messenger that conveys a signal from the ER to the mitochondria.

The siRNA vectors targeted against the PUMA and NOXA genes also inhibited TG-induced apoptosis. These genes induce apoptosis in response to DNA damage or to the overexpression of p53, but there are no reports that NOXA regulates ER stress-induced apoptosis. Moreover, Reimertz et al. (23) suggested that PUMA might be both sufficient and necessary for ER stress-induced apoptosis. To identify components of the apoptotic pathway downstream of the BH3-only proteins, we investigated the activation of the caspase cascade in cells that had been transfected with siRNA vectors directed against the gene for caspase-8, NOXA, and PUMA (Fig. 3). The siRNA vectors against PUMA or NOXA blocked the activation of caspase-9 but failed to prevent the processing of caspase-3 and Bid. These observations indicated that NOXA and PUMA act upstream of a mitochondrial pathway (an intrinsic pathway) but are not part of the extrinsic pathway that is initiated by activation of cell surface death receptors and subsequent direct cleavage of caspase-3 by caspase-8. By contrast, the siRNA vector directed against the gene for MAP3K8 suppressed the activation of the genes for ERK1/2.

Numerous studies, including “knockout” experiments, have indicated that individual BH3-only proteins have specialized physiological roles. For example, Bim plays a role in the cytotoxic response of lymphocytes to cytokine deprivation and to taxol, whereas Bmf seems to be required for anoikis (apoptosis triggered by loss of contact with the extracellular matrix) (24). Our data demonstrated the involvement of several BH3-only proteins in the apoptotic pathway and showed that each of these BH3-only proteins is essential for the induction of apoptosis, suggesting the possible importance of the coordination of the activities of several BH3-only proteins in apoptosis.

**Protein Kinases**—Our screening revealed the involvement of members of the mitogen-activated protein kinase (MAPK) family in TG-induced apoptosis. MAPKs regulate the activity of transcription factors or downstream kinases by phosphorylation, thereby controlling a variety of physiological processes. MAPKs can be classified broadly into three categories: the
ERKs, the c-Jun amino-terminal kinase (JNK), and the MAPK p38 (p38). In our screening, we identified siRNA vectors that targeted genes for ERK2 (MAPK1), MAP2K2, and MAP3Ks (MAP3K3, MAP3K6, and MAP3K8), and we identified these genes as genes that might be involved in TG-induced apoptosis (Table I).

To confirm the involvement of the ERK pathway in TG-induced apoptosis, we examined the activation of ERK1/ERK2 by Western blotting with a phospho-ERK-specific antibody. As shown in Fig. 4A, ERK1/ERK2 was activated 1 h after the start of treatment of cells with TG. Furthermore, suppression of the expression of MAP3K8 clearly impaired the activation of ERK after the start of treatment with TG (Fig. 4B). Although the activation of the ERK and JNK/p38 cascades are generally considered to participate in survival and in stress/death signaling, respectively (25), we failed to detect the involvement of JNK/p38 in TG-induced apoptosis in our screening, even though some siRNA vectors targeted to JNK and p38 were identified as anti-apoptotic siRNAs in our screening for genes in other apoptotic pathways. Collectively, our results suggest that the activity of ERKs, mediated by MAP3K8, but probably not JNK/p38, might play a pro-apoptotic role in TG-induced apoptosis.

Transcription Factors—An siRNA vector directed against E2F1 also acted as a suppressor of TG-induced apoptosis (Fig. 5, A and B). E2F1, a member of the E2F family of transcription factors, is known for its involvement of DNA replication during cell proliferation. E2F1 also regulates apoptosis, and a number of E2F1-regulated genes, including genes for ARF, p73, Apaf1, and BH3-only proteins, participate in apoptosis (26–29). The expression of E2F1 is enhanced and E2F1 induces apoptosis in response to DNA damage (30). It seems possible that suppression of the expression of E2F1 might result in the blockage of TG-induced apoptosis. Hershko and Ginsberg (29) showed that ectopic expression of E2F1 enhanced the expression of the pro-apoptotic BH3-only protein PUMA via direct involvement in NIH3T3 cells. As noted above, siRNA vectors directed against PUMA suppressed TG-induced apoptosis, and E2F1 appeared to regulate TG-induced apoptosis via regulation of the expression of PUMA. Reimertz et al. (23) demonstrated that significant induction of PUMA in response to TG, and our Western blotting analysis demonstrated that enhancement of the expression of PUMA occurred after treatment of cells with TG (Fig. 5C). This

**Fig. 5.** Suppression of expression of the E2F1 gene blocked the enhancement of the expression of the PUMA gene in response to TG. A, confirmation of the effects of siRNA vectors directed against the E2F1 gene. HCT116 cells were transfected with siRNA vectors directed against two target sites in the E2F1 gene (lane 2; siE2F1–1–1; lane 3, siE2F1–2–1), or with the siRNA vector directed against Rluc, as a control. During Western blotting analysis, the membranes were probed with antibodies against the indicated proteins. B, surviving cells after treatment with TG (1 μM) for 48 h. The cells were stained with crystal violet. C, expression of the PUMA gene was enhanced by TG. HCT116 cells were treated with 1 μM TG or vehicle (dimethyl sulfoxide, DMSO) for 36 h. During Western blotting analysis, the membranes were probed with antibodies against the indicated proteins. D, HCT116 cells transfected with siRNA vectors directed against E2F1 gene were treated with 1 μM TG for 36 h. Western blotting of cell lysates was performed with antibodies against the indicated proteins. E, a model for TG-induced apoptosis.
enhancement was not observed in cells in which the expression of PUMA was suppressed via the siRNA vector directed against E2F1 (Fig. 5D). These results suggest that endogenous E2F1 might regulate TG-induced apoptosis via regulation of the expression of PUMA.

DISCUSSION

The data presented here, together with those from other studies (31), suggest a model for TG-induced apoptosis, as shown in Fig. 5E. By screening our siRNA expression library, we were able to identify some novel pathways in TG-induced apoptosis in HCT116 cells. We first detected the death receptor/caspase-8 pathway, which seems to induce both the mitochondrial pathway via cleavage of Bid, and the extrinsic pathway, which bypasses the mitochondrial pathway. We also observed the involvement of three BH3 proteins, which might induce the mitochondrial pathway. Transcription factors in the E2F family appeared to enhance the expression of PUMA. The ERK pathway, which was activated transiently 1 h after the start of treatment with TG and is mediated by MAP3K8, might be part of the TG-induced apoptotic pathway. In preliminary experiments, designed to determine hierarchical relationships among these pathways, we found that the ERK pathway does not appear to be located upstream of the death receptor/caspase-8 pathway and the mitochondrial pathway, and it seems likely that the ERK pathway functions as an independent pathway (data not shown).

The most significant advantages of our siRNA expression library as compared with the widely used synthetic siRNA library are as follows. The interferon response in transfected cells can be avoided (32), and the RNAi effect is sustained for a longer period of time than the inhibitory effects of synthetic siRNAs in proliferating cells. Using our siRNA expression library, we demonstrated here the straightforward identification of novel pathways that had not been identified by other available strategies. We have already constructed siRNA libraries directed against the genes for all the kinases and phosphatases in the human genome and against other genes as well. Further analyses using our large libraries of siRNA expression vectors should provide more precise information about various signaling pathways and enhance our understanding of numerous physiological phenomena.

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