Analysis of Double-stranded RNA-induced Apoptosis Pathways Using Interferon-response Noninducible Small Interfering RNA Expression Vector Library*

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We have developed an original vector library that allowed us to exploit the phenomenon of RNA interference but also allowed us to avoid the confounding effects of the interferon response. In the present work, we used our library of small interfering RNA expression vectors to examine the genes involved in apoptosis that was induced by double-stranded RNA. To our surprise, screening of our library revealed two novel double-stranded RNA-induced apoptotic pathways, a JNK/SAPK-mediated mitochondrial pathway and an ERK2-related pathway, both of which appeared to be independent of the serine-threonine protein kinase-dependent caspase pathway. We also found that MST2 and protein kinase Cα both activated the proapoptotic signal mediated by ERK2. The results of our screening process are expected to aid in the use of our library to explore the possibility of the application of RNAi as a practical approach to gene silencing.

There are two types of siRNA expression vector, the tandem type and the hairpin type. The hairpin type (11) has sense and antisense nucleotides in a single chain, connected via an loop sequence, whereas the tandem type (12) has separate sense and antisense sequences. Hairpin-type vectors allow rapid formation of stable hairpin structures with strong suppressive activity. By using a hairpin-type siRNA expression vector, we succeeded in constructing a vector system that was genetically stable and had strong suppressive activity (12).

The induction of apoptosis by dsRNA is remarkable, not only with respect to the interferon response that is associated with RNAi but also as a mechanism by which mammalian cells protect themselves against viral infection. However, the relevant mechanisms are poorly understood. It has been demonstrated that interferon (IFN) and dsRNA induce programmed cell death or apoptosis in mammalian cells (13). When dsRNA or a virus is introduced into a cell, the host cell induces the synthesis of IFN as a defense mechanism. IFN is a type of cytokine that induces the expression of a number of intracellular genes for the prevention of viral invasion and facilitates the apoptosis of infected cells. IFN induces expression of a dsRNA-dependent serine-threonine protein kinase (PKR) (14), which regulates protein synthesis via phosphorylation of the α-subunit of eukaryotic initiation factor 2α (15), with resultant apoptosis. PKR also functions as a signal transducer to mediate the activities of transcription factors, such as nuclear factor κB (NF-κB) (16). PKR might also regulate the activities of apoptosis-related proteins, such as p53 (17) and Fas (18, 19), and it plays a role in the activation of the caspase pathway (20). However, full details of the dsRNA-induced apoptotic network remain to be defined.
In the present study, we attempted to identify components of the dsRNA-induced apoptotic pathway by using our library of siRNA expression vectors. We screened ~700 vectors that were targeted against 241 genes, which included genes for apoptosis-related proteins, kinases, transcription factors, and other proteins, and we identified several interesting genes, for example, genes for proteins in the Bcl-2 family and the mitogen-activated protein kinase (MAPK) superfamily (JNK/SAPK, ERK1/2, and p38 MAPK). We show here that the dsRNA-induced apoptotic pathway is composed of at least three independent pathways as follows: the "classical" PKR-dependent pathway; a JNK/SAPK-mediated mitochondrial pathway; and an ERK2-related pathway. Contrary to a previously accepted hypothesis, ERK2 promoted dsRNA-induced apoptosis.

**EXPERIMENTAL PROCEDURES**

**Construction of siRNA Expression Plasmids**—The construction of siRNA expression plasmids was based on the pcPUR hU6 vector (iGENE Therapeutics, Inc.; www.igenetheapeutics.co.jp). The vector includes a human U6 promoter, a puromycin resistance gene, and BspMI cloning sites. Because hairpin-type siRNA expression plasmids have higher specific activity than tandem-type plasmids, we purchased synthetic oligonucleotides (Takara, Kyoto, Japan) in which sense and antisense nucleotides were connected by an 11-base hairpin loop and formed as a single chain. After annealing, DNA fragments were ligated into the BspMI sites of pcPUR hU6.

**Prediction of Target Sites**—We constructed our own algorithm for the prediction of target sites of RNAi. This algorithm is based on the partial least squares method (12). We used this algorithm to select at least two target sites in a gene of interest.

**Culture and Transfection of Cells and Determination of Cell Viability**—We cultured HeLa S3 cells in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 1% antibiotics. We cultured HeLa S3 cells in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum and 1% antibiotics.

**Use of the siRNA Expression Vector Library Reduces the Interferon Response**—One of the most significant advantages of the siRNA expression library, as compared with synthetic siRNA libraries, is the reduction in the interferon response in transfected cells. Although it had been postulated that the interferon response occurs only in response to long dsRNAs and not to siRNAs, it was demonstrated recently that some short siRNAs could also induce the interferon response (21). Therefore, we examined the interferon response induced by various types of short dsRNA in detail. Fig. 1, A and B, shows a comparison, in terms of the interferon response, between synthetic and vector-derived siRNAs.

In the experiments shown in Fig. 1, we used 50-bp dsRNA against firefly luciferase to sensitize cells. As shown in Fig. 1A, the synthesized (i.e. *in vitro* transcribed) short dsRNA, targeted to the firefly gene for luciferase, apparently repressed the expression of the firefly gene in a dose-dependent manner, but the expression of *Renilla* luciferase was also suppressed, suggesting that a nonspecific effect might have occurred because of the interferon response. By contrast, the same short dsRNA

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Fig. 1. A and B, nonspecific inhibition by synthesized (in vitro transcribed) 50-bp dsRNA and U6 promoter-driven hairpin RNA targeted against firefly luciferase. A, HeLa S3 cells were transfected with 25 ng of firefly luciferase expression vector, 2.5 ng of Renilla luciferase expression vector, and the indicated amounts of synthesized dsRNA. The left panel shows the relative suppression of firefly luciferase (Fluc), standardized by reference to the activity of Renilla luciferase (Rluc). The right panel shows the absolute activities of the firefly and Renilla luciferases. B, HeLa S3 cells were transfected with 25 ng of firefly luciferase expression vector, 2.5 ng of Renilla luciferase expression vector, and the indicated amounts of the 50-bp hairpin RNA expression vector. The left panel and the right panel show the reductions in relative luciferase activity and absolute luciferase activities, respectively. Each experiment was performed in triplicate, and the results are shown as means ± S.E. C, results of TUNEL assays. The two images on the left show cells transfected with dsRNA (poly(I-C)). The yellow arrowheads indicate apoptotic cells. The right pair of images shows untreated controls. D, HeLa S3 cells were transfected with 0.5 μg of poly(I-C) as indicated. PARP and cleaved PARP in cell extracts were detected by Western blotting. Western blotting with actin-specific antibodies was performed to confirm that equal amounts of cell extracts had been loaded on the gel.
transcribed from a U6 promoter (see below for details) inside cells did not induce the nonspecific reduction in the level of Renilla luciferase via the interferon response (Fig. 1B). Furthermore, we found that introduction of mutations (either C to U or A to G) in the stem region significantly reduced the interferon response that was induced by long and short dsRNAs. The extent of induction of the response depended on the sequence of the short siRNA, but it appeared to be advantageous to avoid any possibility of a strong interferon response by use of siRNA expression vectors, rather than synthetic siRNAs, for the construction of libraries. Moreover, this feature was especially important, for example, in attempts to analyze the genes involved in dsRNA-induced apoptosis, which includes the PKR pathway.

Optimization of Conditions for Use of the Library of siRNA Expression Vectors—A useful siRNA expression library should have strong suppressive activity and high genetic stability. Although the hairpin-type siRNA expression vectors had highly suppressive activity, 20–40% of our constructs acquired mutations within the hairpin region when such vectors were introduced into Escherichia coli. We resolved these problems by designing vectors with several C to T (or A to G) point mutations in the sense region. Such vectors could be sequenced without any problems; they did not exhibit reduced suppressive...
activity, and they were genetically stable in bacterial cells. We also attempted to maximize suppressive activity by constructing a loop based on the sequence of micro-RNA and several other parameters (22). Furthermore, we constructed our own algorithm for the prediction of target sites of siRNA expression vectors (12). Our algorithm ensures a high probability of suppressive activity. Our final optimized siRNA expression vector, which included a hairpin-type sequence, had a U6 promoter, mutations in the sense strand, and an optimized loop sequence. Our vector system allowed the generation of genetically stable and very active siRNA expression libraries (12, 22–26). The vectors (the results are shown in Fig. 1B) were constructed according to this strategy with 50-bp stems in order to facilitate comparison between the interferon responses caused by two different dsRNAs (synthetic versus vector-driven). Other siRNA expression vectors used in this study had “regular” 21-bp stems.

Screening of Genes with the Library of siRNA Expression Vectors—To analyze dsRNA-induced apoptosis comprehensively by using our library of siRNA expression vectors, we induced apoptosis by using dsRNA (poly(I·C)). To optimize conditions for screening, we examined two parameters for the induction of apoptosis, the mode of exposure of cells to poly(I·C), and the method used for transfection. After optimization of transfection conditions, we were able to induce cell death effectively by using small amounts of poly(I·C). To confirm that cell death induced by poly(I·C) was apoptosis, we performed TUNEL assays (Fig. 1C). Fluorescence microscopy revealed aggregating nuclei (Fig. 1C, arrowheads) only in cells treated with poly(I·C), and poly(I·C)-treated cells were TUNEL-positive. The microscopic images of cells revealed features typical of apoptosis. In addition, the cleavage of PARP, as detected by Western blotting, indicated that poly(I·C) did actually induce apoptosis in HeLa S3 cells (Fig. 1D).

The activation of PKR is an important feature of dsRNA-induced apoptosis. Therefore, we selected PKR as a positive control. Fig. 2 shows stained cells, microscopic images, and the results of Western blotting analysis for siRNA expression vectors directed against two target sites in the gene for PKR. These observations confirmed that our siRNA expression vectors were able to suppress the expression of an endogenous gene and that our screening method should work in practice. For our screening analysis, we selected 241 specific genes, including genes for pro-apoptotic proteins, kinases, transcription factors, and various other proteins that appear to be related to apoptosis but whose functions are poorly understood. We defined at least two target sites in each gene to check the validity of our choice of targets.

Identification of Genes That Inhibit dsRNA-induced Apoptosis—We transfected HeLa S3 cells with our siRNA expression vectors (Fig. 3) by using one vector per well, and 36 h after transfection, we selected cells for resistance to puromycin by exposure to the drug for 24 h. After puromycin selection, cells were counted and divided equally into three wells of a 48-well plate. Apoptosis was induced by transfection with dsRNA (poly(I·C)). Twenty four hours after induction of apoptosis, we examined the effects of siRNA expression vectors, and we identified several siRNA expression vectors (Table 1), which inhibited the dsRNA-induced apoptosis, such as S2 in Fig. 3. We categorized the genes that inhibited the dsRNA-induced apoptosis to four groups, namely members of the caspase family, members of the Bcl-2 family, kinases, and others. Members of the Bcl-2 family and kinases other than PKR have not been implicated previously as a part of the network that resulted in dsRNA-induced apoptosis.

Gil et al. (16) described how dsRNA activates PKR via the binding of the dsRNA to two dsRNA-binding motifs. They also showed that PKR-mediated apoptosis involves the FADD-caspase 8 pathway (16). In addition to the genes for the classical mediators of dsRNA-induced apoptosis, namely PKR, caspase 8, and caspase 3, we identified many interesting genes that have not been confirmed previously to play roles in dsRNA-induced apoptosis. The involvement of Bid, BAX, VDAC, cytochrome c, caspase 9, and Apaf-1 suggests that the mitochondrial apoptotic pathway might play an important role in dsRNA-induced apoptosis. Moreover, identification of genes for ERK2, JNK/SAPK, p38 MAPK, MAP2Ks, and MAP3Ks suggests that MAPKs might be significant participants in dsRNA-induced apoptosis. Several factors, which we identified using our vector library, might work in concert with each other and represent various networks.

Mitochondrial Apoptotic Pathway and dsRNA-induced Apoptosis—The mitochondrial apoptotic pathway is one of the major pathways to apoptosis and is initiated by various apoptosis-inducing signals. When an appropriate apoptotic signal reached the mitochondria, Bcl-2 proteins mediated an increase in membrane permeability (27), and cytochrome c was released from the mitochondria to the cytoplasm. The released cytochrome c formed a complex with Apaf-1, and this complex activated caspase 9 and prolonged the apoptotic stimulus. The mediator between the apoptotic signal and the mitochondria was generally a Bcl homology 3 domain-containing pro-apoptotic member of the Bcl-2 family. Bid is also a member of the Bcl-2 family, and its expression is induced by caspase 8 (28). Only a few reports (29, 30) have noted a relationship between the Bcl-2 family and the IPN response on the one hand or between caspase 9 and PKR on the other hand. Moreover, there has been no detailed analysis of the association between the mitochondrial apoptotic pathway and dsRNA-induced apoptosis.

In our screening study, many siRNA expression vectors targeted to genes related to the mitochondrial pathway were able
to inhibit the dsRNA-induced apoptosis. As shown in Fig. 4, siRNA expression vectors against genes for Bid, BAX, VDAC, caspase 9, Apaf-1, and cytochrome c appeared to inhibit dsRNA-induced apoptosis. The suppressive activity for each vector was confirmed by Western blotting, as demonstrated also in Fig. 4. All our results together suggest a role for the mitochondrial pathway in dsRNA-induced apoptosis.

To verify that the mitochondrial apoptotic pathway is relevant to dsRNA-induced apoptosis, we performed Western blotting of cytoplasmic fractions isolated from HeLa S3 cells from 0 to 18 h after the induction of apoptosis (Fig. 5A). Western blotting indicated that cytochrome c was released from the mitochondria about 18 h after transfection with poly(I-C). This result demonstrated that the mitochondrial pathway was operative in dsRNA-induced apoptosis. Next, we examined whether the siRNA expression vectors directed against mitochondrial-related genes could block the release of cytochrome c in response to poly(I-C). As shown in Fig. 5B, when we “knocked down” genes for Bid, BAX, or VDAC, the release of cytochrome c from mitochondria was suppressed. These results demonstrated the participation of the mitochondrial apoptotic pathway in dsRNA-induced signaling. Moreover, we were surprised that “knock down” of the genes for PKR and caspase 8 did not affect the release of cytochrome c from mitochondria.

Taken together, these results indicated that dsRNA-induced apoptosis required the mitochondrial pathway, which is independent of the pathway that includes PKR and caspase 8.

MAPK Superfamily and dsRNA-induced Apoptosis—MAPK cascades are ubiquitous signal-transduction modules that are involved in numerous intracellular phenomena (31). There are at least three different MAPK cascades, which include ERK1/2, JNK (also known as SAPK), and p38 MAPK. It is generally accepted that ERK1/2 signaling is involved in cell proliferation (32), whereas the JNK/SAPK and p38 MAPK pathways are activated preferentially by extracellular stresses (33), such as oxidative stresses, radiation, UV light, and tumor necrosis factor-α. These latter pathways play roles in the triggering of apoptotic signals (34, 35).

As shown in Table I, we identified many siRNA expression vectors, targeted against the MAPK superfamily in particular, against genes for MAPK1, MAPK9, MAPK13, and MAPK14 and several types of MKK and MEKK that interfered with dsRNA-induced apoptosis. Thus, MAPK cascades appeared to be critical for dsRNA-induced apoptosis. There are several reports of the activation of p38 MAPK and JNK/SAPK by viral infection or dsRNA. Chu et al. (36) described two dsRNA-mediated pathways, one of which was the PKR-dependent NF-κB pathway, and the other was the PKR-independent JNK/SAPK pathway.
The expression of the kinase pathway upstream of the release of cytochrome c was verified by the preparation of the cytoplasmic fraction, Western blotting was performed with the indicated targets, which inhibited dsRNA-induced apoptosis. After selection with puromycin, cells were treated with poly(I-C). Eighteen hours after the induction of apoptosis, cells were harvested, and the release of cytochrome c was examined. Whole refers to nonfractionated whole-cell extracts. To verify the preparation of the cytoplasmic fraction, Western blotting was performed with VDAC-specific antibody, N1, negative control 1, siRNA expression vector targeted against Renilla luciferase; N2, negative control 2, siRNA expression vector targeted against HCV. C, similar analysis of the kinase pathway upstream of the release of cytochrome c.

Other researchers have noted that dsRNA signaling stimulates the activities of p38 MAPK and JNK/SAPK, with stimulation being dependent on PKR and RNase L (37). Goh et al. (38) reported that PKR is required for activation of p38 MAPK. However, to our knowledge, a role for ERK1/2 in dsRNA-induced apoptosis has not been described previously. It is generally accepted that the activation of ERK1/2 enhances cell survival (32). However, in the present study, the siRNA expression vector directed against ERK2 strongly inhibited dsRNA-induced apoptosis. Therefore, we focused particular attention on the pro-apoptotic effects of ERK2 in dsRNA-induced apoptosis.

**ERK2 Acts as a Pro-apoptotic Factor in dsRNA-induced Apoptosis**—To confirm the role of ERK2 in dsRNA-induced apoptosis, we examined the activation, by phosphorylation, of ERK1/2 in cells after the introduction of dsRNA (poly(I-C); Fig. 6A). We examined the activation of JNK/SAPK over the same time period to validate our assay. Fig. 6A shows the biphasic increases in levels of phospho-ERK1/2 and phospho-JNK/SAPK with time. The mock-transfected control cells did not show any evidence of the activation of JNK/SAPK or of ERK1/2 (data not shown). Thus, it appeared that ERK1/2 was indeed activated by treatment of cells with dsRNA. Next, we confirmed the suppressive activity of the siRNA expression vectors against genes for JNK/SAPK and ERK2 (Fig. 6B). Fig. 6B also shows the inhibition of dsRNA-induced apoptosis by siRNA expression vectors directed against ERK2. As shown in Fig. 6C, no phosphorylated ERK1/2 was detected in cells in the absence of treatment with poly(I-C). The suppression of the expression of ERK2 clearly decreased the level of phosphorylated ERK1/2 specifically, whereas suppression of the expression of JNK/SAPK and p38 MAPK did not affect the phosphorylation of ERK1/2. These results indicated the pro-apoptotic role of ERK2 in dsRNA-induced apoptosis.

To confirm this result, we performed the rescue experiment with an ERK2 expression vector (Fig. 6D), named pcEF9-ERK2-Rescue, which contains three point mutations, to avoid degradation by the ERK2-siRNA (pU6i ERK2), at the target sequence of siRNA expression vector without conversion of amino acids. We established the stable cell lines that overexpress pcEF9-ERK2-Rescue or pcEF9. These cell lines were transfected with the siRNA expression vector targeted against Renilla luciferase (negative control) or ERK2 (pU6i ERK2). Twenty four hours after transfection, cells were selected with puromycin for 24 h. We then induced apoptosis by transfection of poly(I-C). In the control cell line, which was transfected with the empty vector (pcEF9), depletions of ERK2 by pU6i ERK2 resulted in the blockage of apoptosis (Fig. 6D, lower left). In contrast, the ERK2-Rescue stable cell line, which is resistant to pU6i ERK2, rescued the phenotype of apoptosis. Concomitant with the rescue of apoptosis, the expression level of ERK2 was rescued in the ERK2-Rescue stable cell line (Fig. 6D, lower right). These results clearly demonstrate that ERK2 acts as a pro-apoptotic factor in dsRNA-induced apoptosis.

**Identification of Genes That Function Upstream of ERK2**—In our next experiments, we tried to identify factors located upstream of ERK2. After suppression of several selected genes that inhibited dsRNA-induced apoptosis, we induced apoptosis by transfection of cells with poly(I-C). As shown in Fig. 7, determination of levels of phospho-ERK1/2 by Western blotting demonstrated that phosphorylation of ERK1/2 was suppressed when expression of genes for MST2 or PKCα was suppressed. To our surprise, activation of ERK1/2 was not inhibited by suppression of expression of PKR or of caspase 8. These results indicated that the ERK1/2 pro-apoptotic pathway was independent of the PKR-caspase 8 pathway and was instead mediated by MST2 and PKCα.

MST2 belongs to the family of SPS1/STE20-like kinases (39), and its expression is responsive, for example, to cell stress, various cytokines, and oxidative stress. It has been proposed that members of the MST subfamily might activate caspases via the JNK/SAPK pathway (40). Our results demonstrated that MST2 played a pro-apoptotic role by activating ERK1/2 in dsRNA-induced apoptosis. As for PKCα, it has been proposed that this enzyme is involved in the activation of ERK1/2, p38 MAPK, and JNK/SAPK (41). In view of these observations, it seems appropriate that suppression of the expression of PKCα inhibited the phosphorylation of ERK1/2. However, it has also been suggested that the activation of p38 MAPK and JNK/SAPK via PKCα might induce apoptosis, with ERK1/2 being involved in the inhibition of apoptosis. Our observations suggest a pro-apoptotic role for PKCα in dsRNA-induced apoptosis. Thus, both PKCα and MST2 appear to be involved, together with ERK2, in pro-apoptotic signal transmission.

**Three Independent Pathways in dsRNA-induced Apoptosis**—Here we identified several interesting enzymes that have not been associated previously with dsRNA-induced apoptosis. We also identified the connections among these enzymes and cat-
egorized them to two groups: enzymes in the mitochondrion-related apoptotic pathway, and enzymes in the MAPK pathway. As shown in Fig. 5C, we examined the connection between the mitochondrial pathway and the MAPK pathway. After suppression of the expression of ERK2, p38 MAPK, and JNK/SAPK, we monitored the releasing of cytochrome c from mitochondria by Western blotting.

Only JNK/SAPK-suppressed cells failed to release cytochrome c from their mitochondria, whereas suppression of the expression of ERK2 and p38 MAPK had no effect on the releasing of cytochrome c. These results demonstrated that JNK/SAPK acted upstream of the mitochondrial pathway. In addition, as shown in Fig. 5B, suppression of the expression of PKR and caspase 8 did not influence the release of cytochrome c. These results indicated that the pathway from JNK/SAPK to mitochondria is independent of the classical PKR-caspase 8 pathway.

As described above, the ERK2-dependent pro-apoptotic pathway was mediated by MST2 and PKC/9251 and was unrelated to the PKR and JNK/SAPK pathways. We can summarize our data as depicted by the model of the mechanism of dsRNA-induced apoptosis that is shown in Fig. 8. There appear to be at least three independent pathways, all of which appear to be necessary for the induction of apoptosis by dsRNA.

FIG. 6. A, time course of the phosphorylation of ERK1/2 and JNK/SAPK after treatment of cells with poly(I-C). pERK1/2, phosphorylated ERK1/2; pJNK/SAPK, phosphorylated JNK/SAPK. B, inhibition of dsRNA-induced apoptosis by siRNA expression vectors against genes for ERK1/2 and JNK/SAPK. C, the effects of ERK2, JNK/SAPK, and p38 MAPK-targeted siRNA expression vectors on the activation of ERK1/2. Poly(I-C)(–) indicates the mock-transfected control in which no dsRNA (poly(I-C)) was introduced. D, the construct of ERK2 expression vector named pcEF9-ERK2-Rescue, in which three nucleotides were mutated at the target sequence of parental pU6i ERK2, without conversion of amino acids. The phenotypes after the induction of apoptosis were represented by the photographs (lower left). The anti-apoptotic phenotypes of pU6i ERK2-transfected cells were rescued by the introduction of the pcEF9-ERK2-Rescue vector. The Western blotting was performed to confirm that the transfection of pcEF9-ERK2-Rescue could rescue the expression of ERK2 from the degradation of endogenous ERK2 mRNA by the pU6i ERK2 (lower left). The left lane represents protein of the cells, which were transfected with negative control siRNA expression and pcEF9 vectors. The middle lane represents protein of the cells, which were transfected with pU6i ERK2 and pcEF9 vectors. The right lane represents protein of the cells, which were transfected with pU6i ERK2 and pcEF9-ERK2-Rescue.

FIG. 7. Analysis of the pathway upstream of the activation of ERK1/2. Poly(I-C)(–) indicates the mock-transfected control in which no dsRNA (poly(I-C)) was introduced. The siRNA expression vectors targeted against PKCs and MST2 inhibited the activation of phosphorylated ERK1/2 by the introduction of dsRNA (poly(I-C)).

DISCUSSION

By using our library of siRNA expression vectors, we have identified many genes that are associated with dsRNA-induced apoptosis. We selected at least two target sites in each target mRNA by using our original algorithm, and each of the two target sites was often sufficient for suppression of the target mRNA. The average relative efficiency of suppression of endogenous genes was more than 80%, as a result of the use of optimized vectors.

Our screening revealed the unexpected pro-apoptotic role of ERK2 in dsRNA-induced apoptosis. It was proposed previously that ERK1/2 delivers a survival signal, which counteracts the pro-apoptotic effects of the activation of JNK/SAPK and p38 MAPK. Thus we were surprised that ERK2 was implicated in the dsRNA-induced apoptosis, because ERK1/2 has typically been associated with cell survival (42). However, Murray et al. (43) reported that ERK1/2 had pro-apoptotic effects in hippocampal neurons, and there are several reports suggesting a
requirement for ERK1/2 in cisplatin-induced apoptosis of human cervical carcinoma cells and ovarian cell lines (44). Moreover, Lee et al. (45) reported that oxidative stress-induced apoptosis is mediated by the phosphorylation of ERK1/2. Our result was thus in tune with conclusions obtained in studies of other apoptotic pathways. The pro-apoptotic effect of ERK1/2 might be a general phenomenon. We found that MST2 and PKCε-mediated pro-apoptotic effects of ERK2 provide new clues to the mechanism of dsRNA-induced apoptosis, and these effects are completely independent of the established PKR-dependent pathway.

The participation of the JNK/MAPK pathway in dsRNA-induced apoptosis has been reported by several groups. However, the events that occur upstream and downstream of JNK/MAPK remain poorly defined (36–38). To summarize earlier results, it appears that there are p38 MAPK and JNK/SAPK-mediated mitochondrial apoptotic pathways; the p38 MAPK pathway is dependent on PKR, whereas the JNK/SAPK pathway is dependent on PKR and RNase L. In higher vertebrates, IFN mediates the intrinsic antiviral response, in part through the action of RNase L. RNase L, a uniquely regulated enzyme, is activated by 2′,5′-oligoadenylate, which is produced by IFN-inducible and dsRNA-dependent synthetases. Li et al. (46) described how RNA damage caused by RNase L can lead to the activation of JNK/SAPK and apoptosis. However, to our knowledge no events downstream of the activation of JNK/SAPK in dsRNA-induced apoptosis have been reported previously. It has only been reported that JNK/SAPK causes mitochondrial death signaling in UV-induced apoptosis and the neuron growth factor-reduction pathway (47, 48). Our results demonstrate directly and for the first time the existence of a JNK/SAPK-mediated mitochondrial pathway in dsRNA-induced apoptosis.

CONCLUSION

In the present work, we have succeeded in developing an original vector library that has high suppressive activity and specificity and that allows us to avoid the confounding effects of the interferon response of dsRNA. We screened ~700 clones that were targeted against different genes. We propose that there are at least three independent pathways in the dsRNA-induced apoptotic network as follows: the PKR-caspase 8-dependent classical pathway, the JNK/SAPK-mediated mitochondrial pathway, and the ERK2-related pathway. Apoptosis appears to require the operation of all three pathways simultaneously, because suppression of the expression of only one mediator was able to inhibit the induction of apoptosis by poly(I:C).

In the present experiment, we demonstrate the strength of our vector-based RNAi screening technology, which is free from interference by the interferon response, and we show how it can be used for comprehensive analysis of dsRNA-induced apoptotic pathways. Our large scale screening technology is applicable to the entire human genome and should help in the discovery of the functions of vast numbers of functional genes.

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