2′-5′(A) synthetases are a family of interferon-induced enzymes that polymerize ATP into 2′-5′-linked oligoadenylates that activate RNase L and cause mRNA degradation. Because they all can synthesize 2-5(A), the reason for the existence of so many synthetase isozymes is unclear. Here we report that the 9-2 isozyme of 2-5(A) synthetase has an additional activity: it promotes apoptosis in mammalian cells. The proapoptotic activity of 9-2 was isozyme-specific and enzyme activity-independent. The 9-2-expressing cells exhibited many properties of cells undergoing apoptosis, such as DNA fragmentation, caspase activation, and poly ADP-ribose polymerase and lamin B cleavage. The isozenzyme-specific carboxyl-terminal tail of the 9-2 protein was shown, by molecular modeling, to contain a Bcl-2 homology 3 (BH3) domain, suggesting that it may be able to interact with members of the Bcl-2 family that contain BH1 and BH2 domains. Co-immunoprecipitate assays and confocal microscopy showed that 9-2 can indeed interact with the anti-apoptotic proteins Bcl-2 and BclxL, in vivo and in vitro. Mutations in the BH3 domain that eliminated the 9-2-Bcl-2 and 9-2-BclxL interactions also eliminated the apoptotic activity of 9-2. Thus, we have identified an interferon-induced dual function protein of the Bcl-2 family that can synthesize 2-5(A) and promote cellular apoptosis independently. Moreover, the cellular abundance of this protein is regulated by alternative splicing; the other isozymes encoded by the same gene are not proapoptotic.
antibody was raised in rabbit by Biosynthesis Inc. The 17-mer peptide used as the antigen contained residues 355–371 of the protein (7). Rabbit anti-human Bcl-2 antibody was from Pharmingen (San Diego, CA). Mouse monoclonal anti-human Bcl-2 antibody, mouse monoclonal anti-human Bcl-x antibody, and rabbit anti-Flag antibody were from Santa Cruz Biotechnology. Rabbit anti-human Bcl-x antibody was from Transduction Laboratory and mouse monoclonal anti-Flag antibody was from Promega.

**Synthetase Isozymes and Their Mutants**—The isozymes 9-2, 3-9, L3, and F69 have been described before (7, 17, 18). They were all expressed from pCDNA3 vector driven by cytomegalovirus early promoters as Flag-tagged proteins (10). The enzymatically inactive 9-2 mutant, 9-2M, has two Asp residues in the catalytic domain substituted by Ala (9). The 9-2 TM mutant does not form tetramers and is enzymatically inactive (10). The 9-2 DM mutant had residues 345–414 deleted (7). The 9-2 mutant had residues 345–414 deleted (7). The 9-2 TM mutant does not form tetramers and is enzymatically inactive (10). The 9-2 DM mutant had residues 345–414 deleted (7).

**FIG. 1. Expression of the 9-2 mRNA and protein in human HT1080 cells.** A, the alternative splicing pattern that gives rise to the three mRNAs is schematically shown. The thin lines denote the mRNAs, and the rectangles show the proteins. The first 345 residues are identical, and the rest are unique for each isozyme. The arrows show the positions of the primers chosen to amplify portions of the E18 9-2 and 9-2 mRNAs by reverse transcription followed by polymerase chain reaction. B, partial sequences of the two DNA fragments generated by polymerase chain reaction are shown. Only the sequences at the ends and the splice junctions are given. C, the amino acid sequences of the carboxyl-terminal regions of the three isozymes after the alternative splice point are shown. In the 9-2 sequence, the underlined peptide was used to raise an isozyme-specific antibody. D, expression of the 9-2 protein in IFN-treated human HT1080 cells. Extracts of insect cells expressing the 9-2 protein (lanes 1 and 4) and HT1080 cells transfected with E18 (lanes 2 and 5) and 3-9 (lanes 3 and 6) were analyzed by Western blotting with a common antibody (lanes 1–3) or the new 9-2-specific antibody (lanes 4–6). The 9-2-specific antibody was used to analyze extracts of 1000 units/ml IFN-β-treated (lane 7) and untreated (lane 8) HT1080 cells, after enriching them by poly(I)-poly(C) agarose chromatography.
assay except that the buffer contained 10 mM Tris-Cl, pH 8.0, 150 mM NaCl, 0.2% Nonidet P-40, 1% SDS, 10% glycerol, and 2 mM phenylmethylsulfonyl fluoride. Proteins were analyzed by 10% polyacrylamide gel electrophoresis and a polyclonal Lamin B antibody.

DNA Ladder Assay—Cells at 72 h posttransfection were scrapped out and collected by centrifugation. The cell pellet was incubated with 0.2 mg/ml proteinase K in 500 μl of buffer (100 mM Tris-Cl, pH 8.5, 5 mM EDTA, 200 mM NaCl, 0.2% SDS) at 37 °C. The DNA was precipitated with an equal volume of isopropanol. The precipitated DNA was treated with 0.1 mg/ml RNase A at 37 °C, analyzed on a 2% agarose gel, and stained with ethidium bromide.

Cytochrome C Release Assay—For assaying release of cytochrome C from mitochondria to cytosol, cells were washed with chilled PBS, scraped off the plate in PBS, and harvested by centrifugation. The cell pellet was washed once with PBS and then suspended in 3 volumes of a buffer containing 20 mM HEPES, pH 7.4, 10 mM KCl, 1.5 mM MgCl2, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 10 mM phenylmethylsulfonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, and 250 mM sucrose (29). After chilling on ice for 3 min, the cells were disrupted by 25 strokes in a glass homogenizer. The extract was centrifuged twice at 2500 × g to remove unbroken cells and nuclei. Mitochondria were then pelleted from the supernatant by centrifugation at 12,000 × g for 30 min. The supernatant was removed and filtered successively through 0.2- and 0.1-μm Ultrafree filters (Millipore) to produce the cytosolic fraction. Equal amounts of cytosolic protein were electrophoresed by 12% SDS-polyacrylamide gel electrophoresis and Western blotted with a monoclonal antibody to cytochrome C (Pharmingen) at a 1:5000 dilution followed by a secondary goat anti-mouse horseradish peroxidase-conjugated antibody at a 1:2000 dilution. The secondary antibody was detected by enhanced chemiluminescence.

Molecular Modeling of the BH3 Domains—Amino acid sequences of the BH3 domains of 9-2, BclxL, Bad, and other proapoaptotic family members were aligned using the homology module of Insight II (Molecular Simulations, Inc.). The secondary structures were determined using the FHD see program at predict protein server. Molecular modeling of the 9-2 and the Bad BH3 domains was done using Insight II. For this modeling, the BH3 domain of BclxL was as taken on the template to construct the backbone conformations of the two test BH3 domains.

In Vitro Protein-Protein Interaction Assays—In vitro coupled transcription-translation systems were used for synthesizing radiolabeled Flag-tagged proteins 9-2, its mutants, Bcl-2, and BclxL individually. Equal amounts of the interacting proteins were mixed in the binding buffer containing 10 mM Tris-Cl, pH 8.0, 137 mM NaCl, 0.1% Nonidet P-40, 10% glycerol, and 2 mM phenylmethylsulfonyl fluoride and incubated at 4 °C for 1 h. 9-2 proteins were immunoprecipitated with anti-Flag antibody bound to agarose (9). After washing twice with the binding buffer containing 250 mM NaCl, the bound proteins were analyzed by polyacrylamide gel electrophoresis and autoradiography.

Confocal Microscopy for Protein Co-localization—Flag-tagged 9-2 was transfected, and cells were fixed with 4% paraformaldehyde, permeabilized in 0.1% Triton X-100, washed, and incubated in blocking buffer and then with the primary antibodies. As primary antibodies, rabbit anti-Flag antiserum at a 1:250 dilution and mouse monoclonal anti-Bcl-2 antiserum at a 1:500 dilution were used. As secondary antibodies, fluorescein isothiocyanate-conjugated anti-rabbit goat antimouse serum and Texas Red-conjugated anti-mouse goat antiserum were used at a 1:2000 dilution. The green and red colors were detected by confocal fluorescent microscopy (13).

RESULTS

Expression of the 9-2 Isozyme in Human Cells—We originally cloned the 9-2 isozyme of 2-5(A) synthetase from a mouse cDNA library along with another isozyme, 3-9 (7). The sequence of 3-9 was almost identical to that of E16, a human isozyme, whereas all of 9-2 sequence was contained within that of E18, an alternatively spliced sister of E16. Thus, we proposed that the mRNAs for E16/3-9, E18, and 9-2 arise from the same primary transcript by alternative splicing (Fig. 1A). As a result, all three proteins contain an identical 345 residues at their amino termini and various numbers of unique residues at their carboxyl termini after the alternative splice junction. Because we are interested in determining specific cellular functions of these and other isozymes of 2-5(A) synthetases, we have been expressing them individually in human cell lines. Because we have been using human cells for expression analysis and the E16 and E18 isozymes were originally cloned from human cells, it was important to establish that the 9-2 isozyme is also expressed in human cells. Results presented in Fig. 1 demonstrate the existence of the 9-2 isozyme in IFN-treated human HT1080 cells. To test the existence of the 9-2 mRNA, RNA from IFN-treated cells was used for reverse transcription-polymerase chain reaction using appropriate primers. The sense primer was from the common region just upstream of the splice junction, and the antisense primer was from the region common to
the E18 and 9-2 mRNAs placed right at the translation termination point of the putative 9-2 protein (Fig. 1A). As expected, two pieces of DNA were amplified from RNA of IFN-treated cells (data not shown). Their sizes of 405 and 307 base pairs matched exactly the sizes expected of E18 mRNA and 9-2 mRNA, respectively. None of these DNAs were obtained without reverse transcription. The two cDNAs were completely sequenced, confirming the identities of the E18 and 9-2 mRNAs and the encoded proteins (Fig. 1, B and C). This portion of the human 9-2 sequence was identical to that of the mouse 9-2. The E18 sequence had two amino acid differences: a Thr to Ala and a Thr to Arg substitution as compared with the published E18 sequence (6). Induction of 9-2 mRNA could be detected as early as 2 h after IFN treatment of cells (data not shown). These data demonstrated that the alternatively spliced 9-2 mRNA exists, albeit at a low level, in IFN-treated human HT1080 cells.

Fig. 3. Apoptotic properties of different synthetase isozyme and 9-2 mutants. A, different isozymes. 300 protein-expressing cells were scored for TUNEL positivity. Bar 1, 9-2; bar 2, E-18; bar 3, 3-9; bar 4, L-3; bar 5, P69. Results are presented as percentage of cells undergoing apoptosis. The inset shows the levels of expression of the corresponding proteins in the cell extracts. B, apoptosis of cells expressing two enzymatically inactive mutants of 9-2: 9-2M, a mutant in the catalytic center (9) and 9-2 TM, an oligomerization-defective mutant (10). Assay was done as in Fig. 2A. C, quantitation of apoptosis by different mutants of 9-2: bar 1, WT 9-2; bar 2, 9-2M; bar 3, 9-2 TM; bar 4, 9-2 DM, missing the carboxyl-terminal tail; bar 5, 9-2 BH3M, a mutant in the BH3 domain. The inset shows the levels of protein expression.
wanted to investigate the underlying mechanism. Results shown in Fig. 2 demonstrated that it was due to cellular apoptosis. 9-2-expressing cells were clearly TUNEL-positive, an indication of the presence of multiple ends of DNA produced by apoptosis-associated DNA fragmentation (Fig. 2A). In contrast, cells expressing the sister isozyme 3-9 were not TUNEL-positive. This was not due to a lower level of 3-9 expression; Western blotting of the cell extracts showed that 3-9 was expressed better than 9-2 (Fig. 2B).

In the next series of experiments, the 9-2-mediated apoptotic process was characterized further. For this purpose, 9-2 or 3-9 expression vectors were co-transfected with an expression vector of the cell-surface marker, CD20. CD20-expressing cells were sorted by fluorescence-activated cell sorter, cultured for a day, and used for biochemical analyses. As expected, DNA laddering assay showed DNA fragmentation in cells expressing 9-2 but not in cells transfected with 3-9 (Fig. 2F). Cellular apoptosis is often associated with the cleavage of the nuclear proteins PARP and Lamin B. Such cleavages were detected by Western blotting appropriate cell extracts with antibodies to the two proteins (Fig. 2, C and D). As expected, the 115-kDa PARP was cleaved to an 85-kDa derivative in cells expressing 9-2 but not in those expressing 3-9 (Fig. 2C). Lamin B (70 kDa) cleavage gives rise to a characteristic 35-kDa fragment (Fig. 2D). Similar to PARP, Lamin B was also cleaved specifically in 9-2-expressing cells. DNA fragmentation and protein cleavages in apoptotic cells are preceded by the activation of specific caspases. Such caspase activation was measured in the extracts of 9-2-expressing cells using caspase-specific substrates and inhibitors. Caspase 3 was highly activated in 9-2-expressing cells (Fig. 2E) but not in 3-9-expressing cells. In contrast, caspase 1 was not activated in these extracts, demonstrating the specificity of caspase activation in 9-2-expressing cells. Another hallmark of cells undergoing apoptosis is the release of cytochrome C from mitochondria to cytoplasm. In 9-2-expressing cells, cytosolic cytochrome C was readily detected (Fig. 2G). As expected, no cytochrome C was present in the cytoplasm of vector-transfected cells, but a large amount of it was present in the cytoplasm of cells transfected with Bax, a known proapoptotic protein (Fig. 2G). This series of experiments demonstrated that 9-2 expression in cells causes cytosolic cytochrome C release and caspase activation followed by PARP and Lamin B cleavage and DNA fragmentation, a series of events known to occur in most cells undergoing apoptosis.

 Isozyme-specific and Enzyme Activity-independent Apoptosis Caused by 9-2—Once it was firmly established that 9-2 could cause apoptosis, other 2-5(A) synthetase isozymes were tested for this effect. These proteins were expressed by transfection, and individual cells were stained for protein expression and TUNEL positivity. Of 300 9-2-expressing cells, 225 (75%) were TUNEL-positive (Fig. 3A, bar 1). The corresponding numbers for the two sister isozymes, E18 and 3-9, were 4 and 3, respectively (Fig. 3A, bars 2 and 3). No cells expressing another small isozyme, L3, or the medium isozyme P69 were TUNEL-positive (Fig. 3A, bars 4 and 5). Western blotting showed that all isozymes were expressed at similar levels (Fig. 3A, inset). Thus, the proapoptotic activity was an exclusive property of the 9-2 isozyme.

Because all isozymes could synthesize 2-5(A) and this was the only known activity of this class of proteins, there was no obvious explanation of the proapoptotic activity of the 9-2 protein. The first indication that we may have uncovered an independent activity came from testing the effect of 9-2 expression in RNase L−/− fibroblasts (a gift of Robert Silverman). These cells were killed as efficiently as the WT cells by 9-2, indicating that 2-5(A) activation of RNase L was not required for the
apoptotic effect (data not shown). This prompted us to test whether the enzymatic activity of the 9-2 protein was dispensable as well. We had previously generated an enzymatically inactive mutant of 9-2, 9-2M, in which two Asp residues at its active center were replaced by Ala residues (9). This mutant of 9-2 was active in causing apoptosis in the HT1080 cells as measured by TUNEL assay (Fig. 3B). Another mutant of 9-2, 9-2 TM, is also enzymatically inactive because it is a monomer (10). This mutant retained the proapoptotic activity as well (Fig. 3B). The two enzymatically inactive mutant proteins were expressed to the same levels as the WT protein (Fig. 3C). When their apoptotic activities were quantitated, 9-2M killed 67% of the cells and 9-2 TM killed 61% of the cells as compared with 75% killing by the WT protein (Fig. 3C, bars 1–3). These results clearly established that the proapoptotic activity of 9-2 was independent of its ability to synthesize 2-5(A) and must be mediated by a different pathway.

A Putative BH3 Domain in 9-2—Because the enzymatic activity of 9-2 was not required for its apoptotic activity, we searched for an alternative biochemical explanation for its apoptotic action. A possible lead came from the observation that the 9-2 protein may contain a putative Bcl-2 homology domain 3. This domain was present in the isozyme-specific carboxyl-terminal tail of the protein between residues 372 and 393 (Fig. 4A). When compared with the sequences of the BH3 domains of several members of the Bcl-2 family, all of them, including 9-2, contained predicted α-helical structures in this region. To probe their structures further, molecular modeling studies were done using the known structure of the BH3 domain of BclxL as the template (Fig. 4B). The BH3 domain of BclxL is known to be an amphipathic α-helix. The BH3 domain of Bad and the putative BH3 domain of 9-2 assumed very similar structures when the BclxL residues were replaced by the corresponding residues of the two other proteins. The hydrophilic surface of the BclxL BH3 domain contains two charged residues, Gln and Asp, and the corresponding hydrophobic surface contains two residues, Leu and Phe, that are known to be required to maintain the function of this domain. The Leu, Phe, and Asp residues were conserved in similar locations in the BH3 domains of 9-2 and Bad as well, whereas the Gln residue was replaced by Lys in 9-2 and by Arg in Bad (Fig. 4B). These analyses strongly suggested that the 9-2 protein contains an authentic BH3 domain.

Interaction of 9-2 with Bcl-2 and BclxL—Because the 9-2 protein appears to have a BH3 domain, it may exert its proapoptotic activity by binding to the anti-apoptotic proteins Bcl-2 and BclxL. These proteins contain the BH1 and BH2 domains that form a pocket to which certain BH3 domains can bind. This possibility was tested in the next series of experiments. In the first experiment confocal microscopy was used to examine whether the subcellular location of 9-2 partially overlaps with those of Bcl-2 (Fig. 5A). The distribution of 9-2 is shown in green and that of Bcl-2 in red. When the green image was overlain with the red image, many yellow areas were observed, indicating that subpopulations of 9-2 are located in the same compartments where Bcl-2 resides in cells.

Physical interaction of 9-2 with Bcl-2 and BclxL was tested by co-immunoprecipitation experiments. Endogenous 9-2 was induced in cells by interferon treatment, and Bcl-2 or BclxL was immunoprecipitated from cell extracts. 9-2 was co-immunoprecipitated with both proteins but not with preimmune serum (Fig. 5B).

Importance of the BH3 Domain—To further test our hypothesis that the putative BH3 domain of 9-2 mediates its interaction with Bcl-2 and BclxL, specific mutations were introduced in this region. In the mutant 9-2 BH3M, the two hydrophilic conserved residues, Leu and Phe, and the two hydrophilic
conserved residues, Lys and Asp, in 9-2 (Fig. 4B) were replaced by Ala because similar mutations are known to perturb the functions of the BH3 domain of BclxL. A deletion mutant, 9-2 DM, missing all of the 9-2-specific carboxyl-terminal tail and the 9-2 BH3M mutant were tested for their ability to bind to Bcl-2 and BclxL. When in vitro translated proteins were mixed, both Bcl-2 (Fig. 6A, lanes 1–3) and BclxL (Fig. 6A, lanes 4–6) co-immunoprecipitated with WT 9-2 but not with either mutant. Similarly, when the 9-2 proteins were expressed in cells by transfection, only the WT 9-2 protein co-immunoprecipitated with Bcl-2 or BclxL (Fig. 6B, lanes 1–3). The above experiments established that the interaction of 9-2 with Bcl-2 and BclxL is mediated by its BH3 domain. The contribution of that interaction to the apoptotic activity of 9-2 BH3 mutants. Cells expressing 9-2 DM or 9-2 BH3M were analyzed for TUNEL positivity as described in Fig. 2A.

**DISCUSSION**

We have reported here the identification of a new apoptotic regulatory protein. 9-2 now joins the list of BH3-domain-only proapoptotic mammalian proteins of the Bcl-2 family, which includes Bad, Bid, Bim, Bik, Blik, and Hrk (20, 21). The authenticity of the 9-2 BH3 domain was first verified by molecular modeling exercises using the known structure of the BclxL BH3 domain as the template (22). In this experiment, we also modeled the BH3 domain of Bad, and all three BH3 domains assumed an amphipathic α-helical structure (Fig. 4). Although there was little sequence conservation among the three BH3 domains, the putative structures were remarkably similar. On the hydrophilic side of the helix, a Leu and a Phe residue are conserved two helical turns apart in all of them. Similarly, on the hydrophilic side, an acidic residue, Asp, in all three proteins is two turns away from a basic residue that is different in the three proteins: Lys in 9-2, Arg in Bad, and Gln in BclxL. The functional importance of these residues of 9-2 was demonstrated by their replacement with Ala, which caused a loss of its ability to interact with Bcl-2 and BclxL and to promote apoptosis. The contributions of other residues of the 9-2 BH3 domain in maintaining its functional integrity remain to be evaluated. A detailed mutational analysis of the BH3 domain of Bak has shown that many residues influence its binding affinity for BclxL (22). Such demanding requirements of specific residues are reflected in the fact that all BH3 domains do not interact with all BH1-BH2 pockets. For example, BclxL itself does not form an intermolecular homodimer. It will therefore be necessary, in the future, to examine the full repertoire of Bcl-2 family proteins with which 9-2 interacts.

Three features of the mode of synthesis of 9-2 and its function are unique (Fig. 7). First, it is the product of an IFN-inducible gene and the first IFN-induced protein of the Bcl-2 family. It is worth pointing out that other 2-5(A) synthetase isozymes have been shown to be induced by a variety of agents, such as dsRNA, tumor necrosis factor, IFN-γ, epithelial growth factor, nerve growth factor, and platelet-de-
The 9-2 protein is physiologically relevant. Clearly, in the cause apoptosis, indicating that the observed apoptotic effect equally well, other isozymes and some mutants of 9-2 did not the wild type 9-2 protein (see Fig. 3). Although expressed thetase and various mutants of the 9-2 at the same level as infection may cause overexpression of a protein, one has to be induces hundreds of other proteins as well. Because trans-
known way to induce it is by IFN treatment of cells, which express it by transfection of the cDNA because the only other
activity of 9-2 by dsRNA cannot be ruled out, it definitely
Although potential up- or down-regulation of the apoptotic
proteins are usually triggered by other events, not their synthe-
such apoptosis is mediated by the induction of a high level of the 9-2 protein, a scenario that can be experimentally tested. What is unique about the 9-2-mediated apoptosis as com-
pared with the two other proapoptotic IFN-induced systems, protein kinase, RNA-dependent and RNase L (2), is that no activator, such as dsRNA, is required for triggering it. Thus, it is conceivable that the 9-2 protein is used for tissue remodeling in interferon-unrelated physiological regulations. It is pertinent in this context to note that very high levels of 2-5(A) synthetase are expressed in resorbing chick oviducts (27) and partially hepatectomized livers (28).

Acknowledgments—We thank Alex Almasan and Robert Silverman for valuable reagents and helpful discussion. Thanks are also due to Guan Chen, Yoshihiro Sokawa, Amy Raber, Judy Drazba, Jim Lang, and Shen-Shu Sung.

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A Specific Isozyme of 2′-5′ Oligoadenylate Synthetase Is a Dual Function Proapoptotic Protein of the Bcl-2 Family
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doi: 10.1074/jbc.M100496200 originally published online April 25, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M100496200

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