MOLECULAR AND CELLULAR BIOLOGY, Apr. 1994, p. 2361–2371
0270-7306/94/$04.00+0

Vol. 14, No. 4

The *gadd* and *MyD* Genes Define a Novel Set of Mammalian Genes Encoding Acidic Proteins That Synergistically Suppress Cell Growth

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Received 6 October 1993/Returned for modification 8 December 1993/Accepted 3 January 1994

A remarkable overlap was observed between the *gadd* genes, a group of often coordinately expressed genes that are induced by genotoxic stress and certain other growth arrest signals, and the *MyD* genes, a set of myeloid differentiation primary response genes. The *MyD116* gene was found to be the murine homolog of the hamster *gadd34* gene, whereas *MyD118* and *gadd45* were found to represent two separate but closely related genes. Furthermore, *gadd34/MyD116*, *gadd45*, *MyD118*, and *gadd153* encode acidic proteins with very similar and unusual charge characteristics; both this property and a similar pattern of induction are shared with *mdm2*, which, like *gadd45*, has been shown previously to be regulated by the tumor suppressor p53. Expression analysis revealed that they are distinguished from other growth arrest genes in that they are DNA damage inducible and suggests a role for these genes in growth arrest and apoptosis either coupled with or uncoupled from terminal differentiation. Evidence is also presented for coordinate induction in vivo by stress. The use of a short-term transfection assay, in which expression vectors for one or a combination of these *gadd/MyD* genes were transfected with a selectable marker into several different human tumor cell lines, provided direct evidence for the growth-inhibitory functions of the products of these genes and their ability to synergistically suppress growth. Taken together, these observations indicate that these genes define a novel class of mammalian genes encoding acidic proteins involved in the control of cellular growth.

Growth arrest is an important response to genotoxic stress in both eukaryotic and prokaryotic cells. DNA damage can activate a variety of cell cycle checkpoints in many eukaryotic cells and, in some cells, trigger apoptosis (discussed in reference 22). In mammalian cells, the *gadd* (growth arrest and DNA damage-inducible) genes were originally isolated on the basis of rapid induction by UV radiation in Chinese hamster ovary (CHO) cells but have subsequently been found to be induced by a wide variety of DNA-damaging agents and certain other growth arrest treatments (16). In CHO cells, these genes were rapidly and coordinately induced by the alkylating agent methyl methanesulfonate (MMS) and more slowly induced by growth arrest treatments, such as medium depletion (starvation). Time course and dose-response experiments suggest that these genes are often coordinately induced by a variety of agents. The overexpression of *gadd45*, *gadd153*, and *gadd34* in newborn mouse liver from the lethal c^{14Cos}/c^{14Cos} mutant compared with wild-type (wt) control animals also supports the assertion that they are coordinately regulated (16). The *gadd153* and *gadd45* genes are of particular interest because their expression has been detected in virtually every mammalian line and tissue examined and because their response to genotoxic agents, in particular MMS, has been seen in all cells tested.

The only *gadd* gene member with a known function is *gadd153*. In particular, a new member of the C/EBP family of transcription factors, CHOP-10, has recently been shown to be the murine homolog of the hamster and human Gadd153 proteins (41). Expression of the C/EBP proteins has been associated with a variety of important cellular events, including growth control processes such as differentiation (discussed in reference 41). Heterodimers of CHOP-10 and other C/EBP proteins were unable to bind to a known C/EBP-binding site in DNA. These results suggest that CHOP-10 may act as a negative modulator of the activity of C/EBP-like proteins in certain growth-arrested cells, although interactions of CHOP-10 with other DNA enhancer elements could also occur. In addition, *GADD153/CHOP-10* has recently been shown to be disrupted in a group of soft-tissue sarcomas with a particular chromosomal translocation; interestingly, a novel breakpoint fusion protein consisting of Gadd153 and a new RNA-binding protein, TLS (translocated in sarcoma), is expressed in these tumors (11).

In the case of the Gadd45 protein, the only known vertebrate sequence with which it has similarity is the murine cDNA clone *MyD118* (1), which will be shown to share appreciable homology at both the DNA and protein levels. The function of *MyD118*, like that of *gadd45*, is unknown. However, it is relevant that it and the other MyD genes were cloned on the basis of their immediate-early activation (in the presence of protein synthesis inhibitor) upon induction of terminal differentiation in M1 myeloblastic leukemia cells (26). In these cells, terminal differentiation results in growth arrest and culminates in programmed cell death (apoptosis). This group contains 10 genes in addition to *MyD118*: transcription factors c-*jun*, *jun-B*,

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jun-D, *egr-1*, and *IRF-1*, as well as *ICAM-1*, H1° histone variant and H3.3 histone variant genes, and two unidentified cDNA clones, *MyD116* and *MyD88*. All of the different *MyD* genes were inducible by interleukin-6 (IL-6) and similar cytokines, and evidence has been presented that they are regulated by multiple mechanisms (26).

Further support for a role in growth control by one or more of the *gadd* genes is recent evidence that the tumor suppressor p53 is involved in their regulation (22, 23, 28, 46). p53 has an important role in at least two growth control responses—the G_1 checkpoint and ionizing radiation (IR)-induced apoptosis. p53 functions as a sequence-specific transcription factor, and many of its effects are probably mediated by the activation of downstream effector genes (46). One such effector gene is *gadd45*, whose response to IR is dependent on normal p53 function in human and rodent cells (22). This gene contains a conserved p53-binding site in its third intron (19). This sequence from the human gene bound p53 and conferred p53 responsiveness to a reporter gene (22). A further connection with the IR-induced G_1 checkpoint is the findings that cells from ataxia telangiectasia (AT) patients are deficient in the normal induction of *GADD45* mRNA (36) and p53 protein (22); these cells are also deficient in the activation of this checkpoint (4, 5). A second effector gene is the oncogene *mdm2*, which probably functions in a negative feedback loop to limit the duration of p53 action (30, 35). Interestingly, as with *GADD45*, *MDM2* induction after IR is strictly dependent on normal p53 function; overexpression of Mdm2 protein in human cells blocked induction of *GADD45* mRNA and the G_1 checkpoint but not p53 protein (7). Thus, one can propose a growth control pathway (7, 22) involving the following: (i) after IR, DNA damage is recognized and the signal is transmitted to the pathway involving the AT gene product(s) at an early step; (ii) p53 is induced by a translational and/or posttranslational mechanism resulting in increased protein and activity; (iii) p53 then acts as a transcription factor to induce *GADD45*, *MDM2*, and possibly other effector genes, which trigger the G_1 checkpoint; and (iv) with increased Mdm2 protein, further p53 transcriptional activity is blocked, allowing cell growth.

Since the *gadd* genes can be induced by agents such as MMS and medium depletion in many cells regardless of p53 status, these responses do not strictly require p53 as is the case for IR. However, there is increasing evidence that p53 may have a cooperative role in such responses. In particular, we have recently found that the spectrum of agents that induce p53 activity, as measured by increased p53 levels and p53 transcriptional activity (47), is very similar to that for *gadd* gene induction. Interestingly, MMS or medium depletion induced p53 activity much more strongly than IR. Second, induction of *GADD45* and *GADD153* by MMS and the small response of *GADD153* to IR were reduced in AT cells compared with normal cells (36). These results suggest a possible role for p53 and the *gadd* genes in the responses to a variety of stresses eliciting growth arrest.

In addition to the genes discussed above, other groups of growth arrest-associated genes such as the *gas* genes (42), *mov10* (31), and the prohibitin gene (13) have also been identified in recent years. Such genes show increased expression during various growth arrest states. For example, the *gas* genes were isolated on the basis of induction in NIH 3T3 cells after serum reduction; *mov10* is a murine gene induced in growth-arrested cells which encodes a GTP-binding protein (31); and the prohibition gene is expressed in differentiated adult tissues, such as liver, and shows reduced expression during growth, such as after partial hepatectomy. No sequence similarities of these genes with the *gadd* genes have been found, and, as will be presented, the responses of the *gadd*/*MyD* group are dissimilar to the responses of these other growth arrest genes

In this work, we reveal a striking overlap between the *gadd* and *MyD* genes. It is also shown that the four gadd/MyD proteins, as well as Mdm2, harbor numerous acidic residues and have a very similar and unusual net negative charge. Analysis of the expression of these four genes in NIH 3T3 cells and M1 myeloblastic leukemia cells highlights their complex regulation and distinction from other growth arrest genes and suggests that the *gadd*/*MyD* genes play a role in growth arrest and apoptosis either coupled with or uncoupled from terminal differentiation. Direct evidence is presented for the synergistic roles that the *gadd*/*MyD* genes play in the suppression of cellular growth.

MATERIALS AND METHODS

Plasmid cDNA clones and DNA sequence determination. The partial-length clone pDDIA34 was originally isolated from CHO cDNA library constructed after hybridization subtraction to enrich for UV radiation-inducible transcripts (15). Nearly full-length hamster clones pXR45m (*gadd45*) and pXR34d (*gadd34*) were isolated from a custom library constructed by Stratagene (La Jolla, Calif.), which has been described previously (36). For *gadd153* studies, a nearly full-length hamster clone, pA5A4 (16), or mouse clone, *CHOP-10* (41), was used. The murine clones *MyD116* (27) and *MyD118* (1) were originally isolated from the M1 myeloid line (26). Other plasmids used included MDM-FL4, a human *MDM2* clone (34), and pC53-SN3, a construct expressing wild-type p53 driven by a cytomegalovirus promoter (47), provided by B. Vogelstein; murine *gas1* and *gas2*, provided by L. Philipson (42); p10-CD7 (murine *mov10*) (31), provided by K. Harbers; pTardin, an initial rat clone for prohibitin (32) provided by D. Danner; pcD51, a murine RCCI (45) clone provided by T. Nishimoto; pA2, a hamster β-actin probe (15); and GAPD, human glyceraldehyde-3-phosphate dehydrogenase (ATCC repository no. 57090). DNA sequencing was performed as described previously (36) on plasmid clones; both strands were sequenced entirely. A portion of the DNA sequencing was performed by Lark Sequencing, Inc. (Houston, Tex.). The sequence of the mouse *gadd45* mRNA was derived from the exon regions of the genomic clone pMG45x1 (19); exon-intron boundaries were conserved between mouse, human, and hamster sequences (data not shown).

For expression in mammalian cells, the pCMV.3 vector, provided by B. Howard, was used and will be described elsewhere (33). pCMV45, pCMV153, and pCMV118 were constructed by PCR cloning of the open reading frame (ORF) region from human cDNA clones for *GADD45* (pHu145b2 [22]), and *GADD153* (pHu175-2A [37]) and from the murine *MyD118* clone described above. PCR amplification of the ORF region was done with a high-fidelity polymerase, Vent (New England Biolabs), and was carried out for only 14 rounds to minimize the chance of mutations; multiple independent clones for each construct behaved similarly. *Hin*dIII or *Xba*I recognition sites were included in the primers, and following PCR, the products were digested and ligated into these sites in pCMV.3 downstream from a pCMV promoter. pCMV45 extended 10 nucleotides (nt) 5′ to the translation start site and 6 nt 3′ to the stop site; the positions were 28 nt 5′ plus 8 nt 3′ and 10 nt 5′ plus 5 nt 3′ for pCMV153 and pCMV118, respectively. pCMVas45 was constructed in a similar manner but included the 5′ nontranslated portion of human *GADD45* mRNA; it was inserted into pCMV.3 in the antisense orienta-

tion. pCMV34 was constructed by ligation of the *Not*I-*Mse*I fragment of pXR34d, including the entire ORF, into the *Not*I-*Xba*I site of pCMV.3 after filling in of the *Mse*I and *Xba*I overhangs with Klenow polymerase (New England Biolabs). pCMV34 included 50 nt of polylinker from pXR34d and 214 nt of nontranslated cDNA 5′ to the ORF and extended 72 nt 3′ of the translation stop site. pCMV.3-p53, which contains the human p53 ORF in pCMV.3, was provided by B. Howard. For selection of stably transfected clones, pSV2neo was used (47).

Cells, cell treatment, and animal studies. The human tumor lines RKO, H1299, and HeLa were grown and treated as described previously (47), as were CHO cells (16), NIH 3T3 cells (14), and M1 cells (26). Primary Syrian hamster embryonal (HEC) were provided by J. Dipaolo (12); they were grown in Dulbecco's modified Eagle's medium with 4.5 g of glucose per liter plus 10% fetal calf serum supplemented with thymidine, hypoxanthine, and pyruvate and were used at passages 6 to 10. All cells were exponentially growing unless otherwise indicated. For medium depletion, HEC and CHO cells were grown to high density and incubated for 2 days without refeeding. Adherent cells were treated with 100 μg of MMS per ml for 4 h as described previously (16). Treatment with prostaglandin A_2 (Calbiochem) was at 36 μM, as described previously (8). In the case of M1 cells, MMS (100 μg/ml), IL-6 (100 μg/ml); Amgen), or lung-conditioned medium (LUCM) at a final concentration of 5% was added to suspension cell cultures at the indicated times (26). Apoptosis was scored when ≥50% of M1 cells appeared apoptotic, as determined by loss of cell viability (trypan blue exclusion) and distinctive apoptotic morphology of May-Grünwald-Giemsa-stained cytospin smears; the latter included small cells, shrunken cytoplasm, condensed nuclei, and fragmented chromatin. Adult male CD rats were injected with 3 mg of lipopolysaccharide (LPS; Sigma) per kg of body weight. Animals were killed at the indicated times by CO_2 asphyxiation, and total liver RNA was prepared as previously described (40).

Transfection conditions. Plasmid DNA was transfected by the calcium phosphate method as described previously (47). Growing cells were seeded in 100-mm-diameter dishes at 2×10^4 to 1×10^5, with two to four plates per point in each experiment; 20 h later, they were cotransfected with 0.5 μg of pSV2neo and either 10 or 15 μg of the indicated pCMV.3 constructs. For each experiment, the total amount of transfected pCMV.3 plasmid(s) was constant for all samples, as was the cell number transfected; controls received pCMV.3 vector containing no insert. Thirty-six hours after transfection, 400 μg of Geneticin (G418; Sigma) per ml was added. The medium was replaced 5 and 10 days after transfection with fresh medium containing G418, and the cells were fixed at 14 days and scored for colonies containing at least 50 cells.

RNA isolation and blot analysis. Cells were lysed in situ with 4 M guanidine thiocyanate; poly(A) RNA was prepared and then analyzed by Northern (RNA) blot analysis or by quantitative dot blot hybridization as described previously (20, 36) for 3T3, HEC, and CHO cells. The relative poly(A)$^+$ content of each RNA sample in the dot blot analyses was estimated by using a labeled polythymidylate probe (20). For analysis of whole-cell RNA from rat liver or M1 cells, 10 μg, as determined by optical density at 260 nm only, was loaded in each lane. Following hybridization with various cDNA probes, the films were exposed for periods during which band intensity was linear with respect to time. Quantitative analysis of autoradiograms of M1 RNA blots was measured at 560 nm with the gel scan program of a Beckman DU7 spectrophotometer as described previously (25). Southern blot analysis of genomic DNA was performed with Hybrisol I solution (Oncor) according to the manufacturer's recommendations.

EMSA. Electrophoretic mobility shift assays (EMSA) were carried out as described previously (22, 47). Briefly, 15 μg of nuclear extract, prepared from control (untreated growing) cells or treated cells, was incubated with a labeled 30-bp fragment from the *GADD45* gene containing the p53-binding site (positions +1569 to +1598). In all samples, 0.2 μg of a monoclonal anti-p53 antibody (PAb421) was added prior to the addition of nuclear extract (22). The samples were then analyzed on a 4% nondenaturing acrylamide gel.

Nucleotide sequence accession numbers. The GenBank accession number of the pXR34d sequence is L28147; that of the mouse *gadd45* sequence is L28177.

RESULTS

Overlap of the *gadd* genes with the *MyD* genes and unusual charge characteristics. To determine the sequence of the Gadd34 protein, a 2,087-nt cDNA clone [excluding the poly(A) tail] for *gadd34* was isolated that matched the original partial-length clone DDIA34 at positions 878 to 1033 (15). It contains a single large ORF encoding a 590-residue protein. As seen in Fig. 1A, there is marked homology with the murine MyD116 sequence, and both the nearly full-length clone and DDIA34 hybridized to the same size bands as MyD116 in genomic Southern blots of restricted human and mouse genomic DNA (data not shown), which indicates that they are encoded by the same gene. The murine protein is 82% similar and 71% identical to its hamster homolog. An interesting feature of the Gadd34/MyD116 proteins is the almost perfect repeats consisting of approximately 3.4 repeats of a 40-residue sequence in Gadd34 and 4.4 repeats of a 39-residue sequence in MyD116. Each repeat contains PEST motifs implicated in rapid protein turnover (39). To compare the murine Gadd45 and mouse MyD sequences, the sequence of the murine *gadd45* mRNA was derived from a murine genomic *gadd45* clone. The murine Gadd45 protein was very similar to the human and hamster (36) homologs, with 95 and 98% homology, respectively. As shown in Fig. 1B, Gadd45 is 57% homologous and 89% similar to MyD118; the two murine proteins are of similar size, and both are highly charged. It is notable that these very similar proteins show no convincing homology with other known sequences from any higher eukaryote (GenBank release 78.0 [August 1993]). Therefore, they define a new gene family whose two known members have been associated with negative growth responses.

Like Gadd45 and Gadd153, the Gadd34/MyD116 and MyD118 proteins are highly charged with many acidic residues; a closer examination reveals an unusually high net negative charge for these proteins and also for Mdm2 (Table 1). When the net charge is normalized to protein size and expressed as net charge percent [$100 \times (K + R - D - E)$/total residues], the values varied by only 30%, from −11.3 to −7.9% in three mammalian species for the four proteins. This strikingly similar charge characteristic is surprising considering that except for Gadd45 and MyD118, none of these proteins show appreciable homology. This charge characteristic is significant since a survey (21) of a large number of proteins from the SWISS-PROT data base revealed that most were not as acidic; e.g., 99% of the human proteins had a net charge percent of ≥ −8.8 and 95% had a net charge percent of ≥ −5.1. An examination of the distribution of acidic residues indicates that they are relatively uniformly distributed throughout, with only one acidic cluster of five or more contiguous residues in Gadd45, MyD118, Gadd153, and Mdm2 and several such clusters in

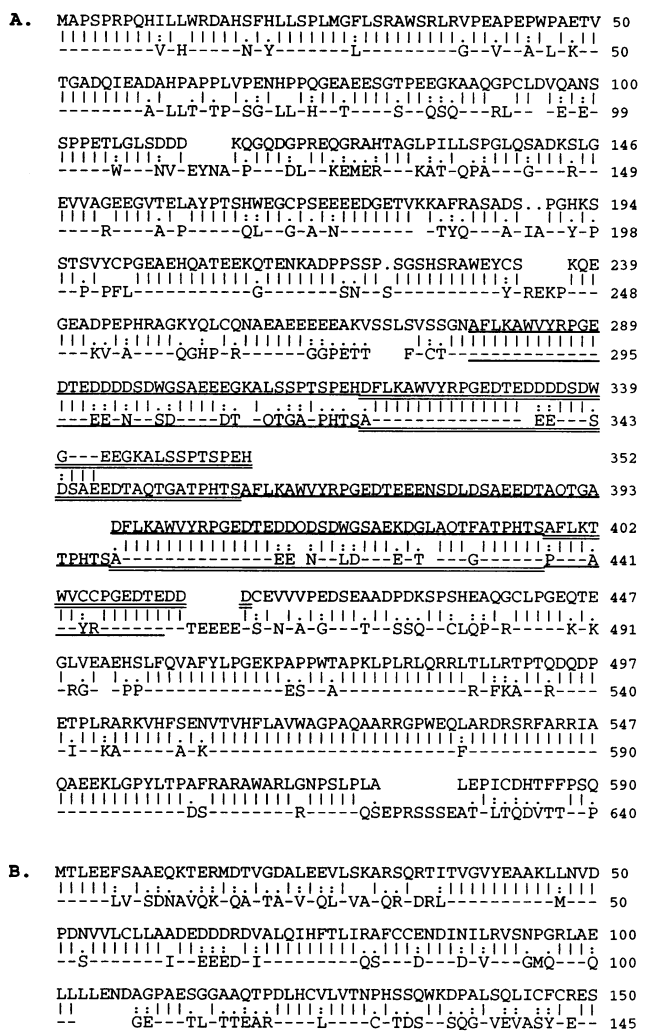

FIG. 1. Homology between Gadd and MyD proteins. (A) Predicted peptide sequence of the hamster Gadd34 protein. The sequence was derived from pXR34d , a nearly full-length cDNA clone containing the entire ORF. The 39- and 40-residue repeats are indicated by alternating single and double underlining. The sequence of mouse MyD116 (27) is compared below (Gappep program of the University of Wisconsin Genetics Computer Group package). |, identity with substitution of dash for one-letter code; :, similarity comparison score of ≥0.5; ., similarity comparison score of ≥0.1. (B) Predicted peptide sequence of the mouse Gadd45 protein, derived from mouse genomic sequence with comparison, with format as in panel A, to mouse MyD118 (1).

Gadd34/MyD116 (data not shown). As will be discussed in more detail below, the unusual charge characteristics are only one of several important properties shared by these growth arrest-associated genes.

Responses of the *gadd* and related genes differ from responses of other growth arrest genes. To determine whether the expression of the *gadd* genes has similarities to expression of other growth arrest-associated genes, expression of various growth arrest-associated genes, including *mdm2*, *gas1*, and *mov10*, was studied in mouse 3T3 cells following growth arrest-inducing treatments. These studies were conducted in this cell line because it is the same line from which the *gas* genes were isolated (42) and because the growth arrest response of the *gas* genes is limited to only certain lines; e.g., the response was lost in a *ras*-transformed 3T3 line (10) and also in CHO cells (data not shown). As shown in Table 2, only the three *gadd* genes plus *MyD118* and *mdm2* were induced by the alkylating agent MMS. The response of *MyD118* was less than that of the *gadd* genes in 3T3 cells, while induction was strong in HEC and CHO cells (Fig. 2A). In the case of *MDM2*, it has been found also to be DNA damage inducible in a variety of human lines by a p53-dependent mechanism (7). In contrast, mRNA levels for *gas1* and *mov10* actually decreased, while the controls β-actin and GAPD showed no appreciable change. *gas2*, prohibitin, and *RCC1* mRNA levels were also not affected by MMS (data not shown); *RCC1* was included because it is a murine gene involved in preventing premature chromosome condensation (45).

It is noteworthy that the responses of the genes in Table 2 to the various growth arrest treatments, other than MMS, highlight the complexity of such responses and differences in their regulation. For example, the three *gadd* genes were more strongly induced by medium depletion (designated "starved") than by contact inhibition and reduced serum, while the patterns of induction were different for *gas1* and *mov10*. The argument could be made that the *gadd* genes respond poorly to normal growth arrest states such as contact inhibition but more strongly to stressful states such as medium depletion whereby one or more vital nutrients have been dissipated. In the case of reduced serum, time course experiments in CHO cells showed variable induction at 24 or 48 h and stronger induction at 72 h, by which time nutrients, such as glucose, may have been depleted (unpublished data). The response to prostaglandin A_2, which inhibits cell growth and reduces viability (8), was restricted to the three *gadd* genes and *MyD118*. Another difference among the five genes encoding acidic proteins was the lack of induction of *MyD118* and *mdm2* by medium depletion (Table 2 and Fig. 2A). Thus, these genes, encoding acidic proteins, on the one hand show coordinate induction by DNA-damaging agents, which distinguishes them from other growth arrest genes, and on the other display differences in expression in response to other growth-inhibitory stimuli, which highlights the complexity of these responses.

TABLE 1. Net charge (K + R − D − E) of predicted proteins

Source	Net charge[a]				
	Gadd45	MyD118	Gadd153/CHOP-10	Gadd34/MyD116	Mdm2
Hamster	−15 (−9.1)		−14 (−8.4)	−61 (−10.3)	
Human	−16 (−9.7)		−18 (−10.7)		−40 (−8.1)
Mouse	−14 (−8.5)	−18 (−11.3)	−14 (−8.3)	−52 (−7.9)	−36 (−7.4)

[a] Each value in parentheses indicates net charge divided by total number of amino acid residues in the protein and is expressed as a percentage; e.g., the net charge of hamster Gadd45 (−15) divided by 165 total residues in this protein is −9.1%.

TABLE 2. Relative expression of growth arrest gene transcripts in 3T3 cells

Treatment[a]	mRNA level with given cDNA probe[b]								
	gadd45	*gadd153*	*gadd34*	*MyD118*	*mdm2*	*gas1*	*mov10*	β-Actin	GAPD
MMS	19.3	10.0	31.8	2.0	3.4	0.3	0.4	0.9	0.9
Confluent	5.0	1.1	1.2	0.7	1.3	5.1	2.2	0.5	1.0
Starved	19.2	22.0	17.9	0.6	1.4	6.3	0.7	0.3	0.7
Starved, MMS[c]	24.6	15.6	75.5	0.8	1.3	0.4	0.1	0.3	0.4
Low serum, 24 h	2.4	1.0	0.9	0.9	1.0	3.6	3.2	0.6	0.7
Low serum, 48 h	2.7	1.5	0.9	1.2	1.3	5.0	5.1	0.5	0.5
PGA_2	8.0	8.0	5.3	2.0	1.1	1.1	1.0	0.8	1.2

[a] Exponentially growing cells were treated with MMS for 4 h and prostaglandin A_2 (PGA_2) for 24 h or were switched to medium containing 0.5% serum for the indicated times; cells were grown to confluence and incubated for an additional 4 days with (confluent) or without (starved) a final medium change 18 h prior to harvest.

[b] The relative abundance of RNA from treated samples was determined by quantitative dot blot hybridization (see Materials and Methods) and normalized to that of untreated, exponentially growing cells isolated at the same time. Increases of >1.5-fold have been shown to be accurately measured with this approach (20).

[c] Treatment was the same as for the sample designated "starved" with the addition of MMS 4 h prior to harvest.

Since *mdm2* is known to be regulated by p53 in a negative feedback loop (30), the p53 status of the 3T3 line used in our studies was determined by EMSA. It has previously been shown that p53 activity, as measured by increased DNA binding to a defined p53 site, is inducible in cells with normal p53 function but not mutant status (22, 44). As shown in Fig. 3, the uppermost band in the lanes from 3T3 cells was clearly inducible by MMS or IR. A band with identical electrophoretic mobility was seen in H1299 cells, which have a null p53 status (47), after transfection with a p53 expression vector. A second inducible band was seen immediately below this band in the mouse line but not the transfected cells; considering that it was not visualized without the addition of anti-p53 antibody (data not shown), it probably represents a second p53-containing complex which may be specific for this rodent line. Multiple noninducible bands with faster mobility in the gel were visual-

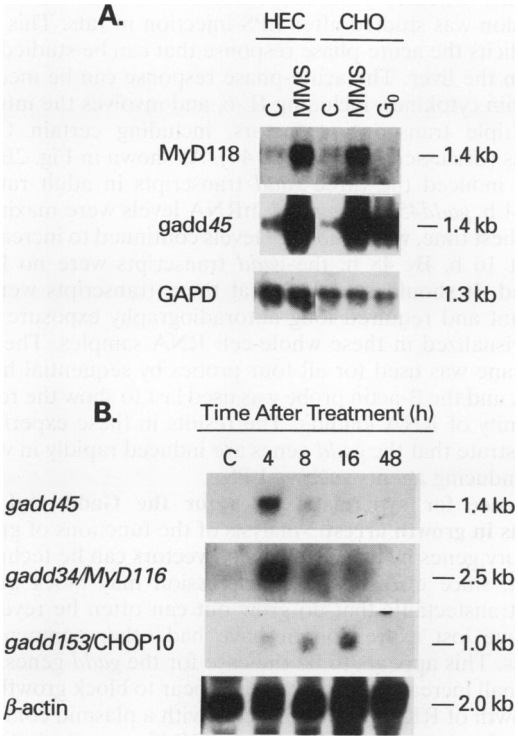

FIG. 2. RNA blot analysis of rodent cells in vitro and in vivo. (A) Expression of *MyD118* and *gadd45* in hamster cells. Equal amounts of poly(A) RNA (1 μg) from HEC and CHO cells were analyzed by Northern blot hybridization with the indicated cDNA probes; samples were from untreated growing cells (C), cells treated with MMS for 4 h, or medium-depleted cells designated (G_0). Only the hybridizing band with its estimated size is shown. (B) Expression of the *gadd* genes in vivo. Approximately equal amounts of whole-cell RNA from rat liver were analyzed as in panel A with the indicated cDNA probes. Liver was obtained from untreated rats (C) or rats sacrificed at the indicated times after injection with LPS (see Materials and Methods).

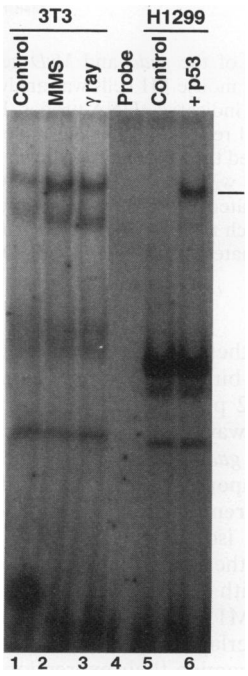

FIG. 3. Induction of p53 in NIH 3T3 cells as determined by EMSA. Equal amounts of nuclear protein from NIH 3T3 or H1299 cells were analyzed as described in Materials and Methods. Samples consisted of untreated cells (lanes 1 and 5) and cells subjected to MMS treatment (100 μg/ml for 4 h; lane 2) or γ-ray treatment (3 h after 20 Gy; lane 3). The sample in lane 6 was transfected with the p53 expression vector pC53-SN3; in the sample in lane 4, no nuclear extract was added. All samples were run on the same gel, and intervening lanes were deleted between the third and fourth lanes of the same autoradiograph. The uppermost band, designated with a hash mark at the right, migrated at the same relative position in the 3T3 and H1299 samples if the slight electrophoretic distortion ("smile") is taken into consideration.

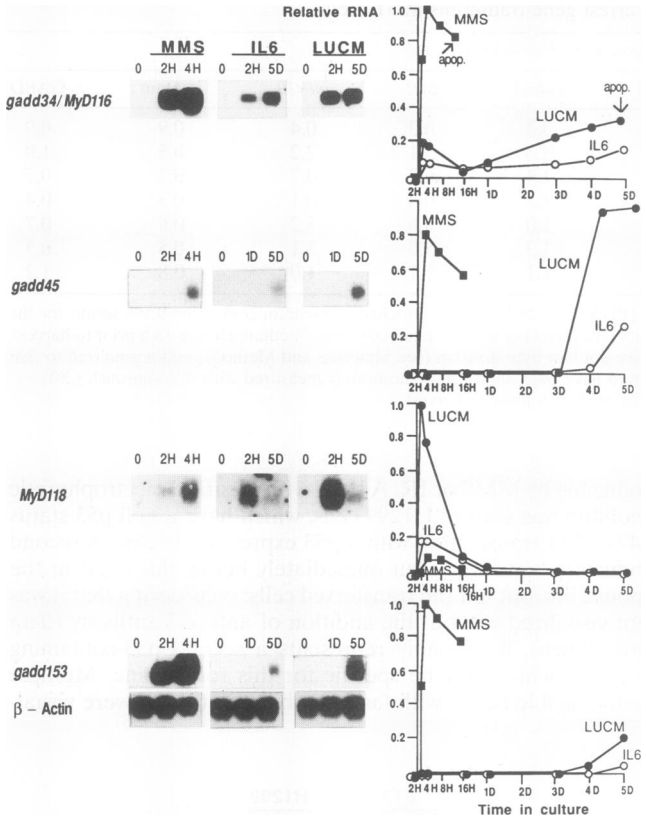

FIG. 4. Expression of the *gadd* and *MyD* genes in myeloid cells. Whole-cell RNA from mouse M1 cells was analyzed by Northern blot hybridization with the indicated cDNA probes. Hybridizing bands are shown on the left, and relative levels determined by scanning densitometry of more detailed time courses are shown on the right. Growing M1 cells were treated with MMS, IL-6, or LUCM for the indicated times; values for untreated cells are shown at time zero. In the top right panel, the time at which the cells showed clear morphologic evidence for apoptosis is designated "apop." H, hours; D, days.

ized in both lines; these probably represent interactions with other cellular DNA-binding proteins. These EMSA results and the data of Table 2 provide evidence for the existence of a functional p53 pathway for this cell line.

Expression of the *gadd* and *MyD118* genes upon induction of growth arrest and apoptosis either coupled with or uncoupled from terminal differentiation. As discussed earlier, the MyD cDNA clones were isolated on the basis of rapid induction without protein synthesis upon induction of terminal differentiation associated with growth arrest that ultimately culminates in apoptosis of the M1 myeloblastic leukemia cell line (26, 43). Considering the overlap between the *MyD* and *gadd* genes, it was of interest to examine their expression in M1 cells following treatment with various growth arrest-inducing agents. Hematopoietic differentiation-inducing agents, such as LUCM or IL-6, induce the entire myeloid terminal differentiation program, including growth arrest and programmed cell death (26, 43). LUCM, the most potent differentiation inducer, contains a variety of cytokines, including IL-6 and leukocyte-inhibitory factor (18). In contrast, apoptosis can also be triggered more rapidly without differentiation by certain agents, including MMS (Fig. 4 and legend).

As shown in Fig. 4, LUCM and to a lesser extent pure recombinant IL-6 rapidly induced *MyD116* and *MyD118* genes, as measured by increased mRNA levels, but not *gadd45* or *gadd153*. However, *gadd45* and *gadd153* were induced at later times following induction of the myeloid differentiation program, near or at the time when the majority of cells were apoptotic. Apoptosis was scored by morphologic changes (see Materials and Methods), but apoptotic DNA ladders were detectable by gel electrophoresis (data not shown). Interestingly, *gadd34/MyD116* showed a biphasic response, while *MyD118* showed only the early *MyD* response. In sharp contrast, following treatment of M1 cells with MMS, which resulted in early apoptosis (by 16 h), all four genes were rapidly induced. The three *gadd* genes can be distinguished from *MyD118* by their stronger response to MMS; in contrast, *MyD118* responded more strongly to LUCM or IL-6 than to MMS. It can be observed that the early induction of both *gadd34/MyD116* and *gadd153* by MMS was stronger than induction by LUCM or IL-6 at any time, while *gadd45*, although exhibiting different induction kinetics with MMS than with LUCM, was induced to similar levels by these agents. Taken together, these observations further highlight the complex regulation of the *gadd/MyD* genes upon induction of growth arrest and apoptosis, either coupled with or uncoupled from terminal differentiation, and are suggestive of multiple, and potentially cooperative, roles in growth arrest, differentiation, and apoptosis.

Expression of the *gadd* genes in vivo. The studies presented to this point have used only cultured cells; therefore, to determine whether such responses can be elicited in vivo, expression was studied after LPS injection in rats. This treatment elicits the acute-phase response that can be studied most easily in the liver. The acute-phase response can be mediated by certain cytokines, including IL-6, and involves the interplay of multiple transcription factors, including certain C/EBP proteins (discussed in reference 40). As shown in Fig. 2B, LPS rapidly induced the three *gadd* transcripts in adult rat liver within 4 h. *gadd45* and *gadd34* mRNA levels were maximal at the earliest time, while *gadd153* levels continued to increase for at least 16 h. By 48 h, the *gadd* transcripts were no longer detected. It should be noted that these transcripts were not abundant and required long autoradiography exposure times to be visualized in these whole-cell RNA samples. The same membrane was used for all four probes by sequential hybridization, and the β-actin probe was used last to show the relative uniformity of RNA loading. The results in these experiments demonstrate that the *gadd* genes are induced rapidly in vivo by stress-inducing agents such as LPS.

Evidence for synergistic roles for the Gadd and MyD proteins in growth arrest. Analysis of the functions of growth-inhibitory genes by using expression vectors can be technically difficult, since even low-level expression may block growth; stable transfectants that do grow out can often be revertants that have lost expression or have had other compensatory changes. This appears to be the case for the *gadd* genes, since even small increases in expression appear to block growth; e.g., the growth of RKO cells transfected with a plasmid containing an 8-kb fragment of the human *GADD45* gene (including 2.5 kbp of promoter) was reduced, and colonies stably expressing the same gene fragment could not be isolated from transfected CHO cells (20a). In an attempt to quantify the growth-inhibitory properties of these *gadd/MyD* genes while circumventing these problems, we used a short-term assay in which expression vectors employing the cytomegalovirus promoter were cotransfected with a selectable marker, pSV2neo, and G418-resistant colonies were scored only 2 weeks later. Studies were performed in human tumor lines with a null p53 phenotype (H1299), in cells with a normal p53 phenotype (RKO),

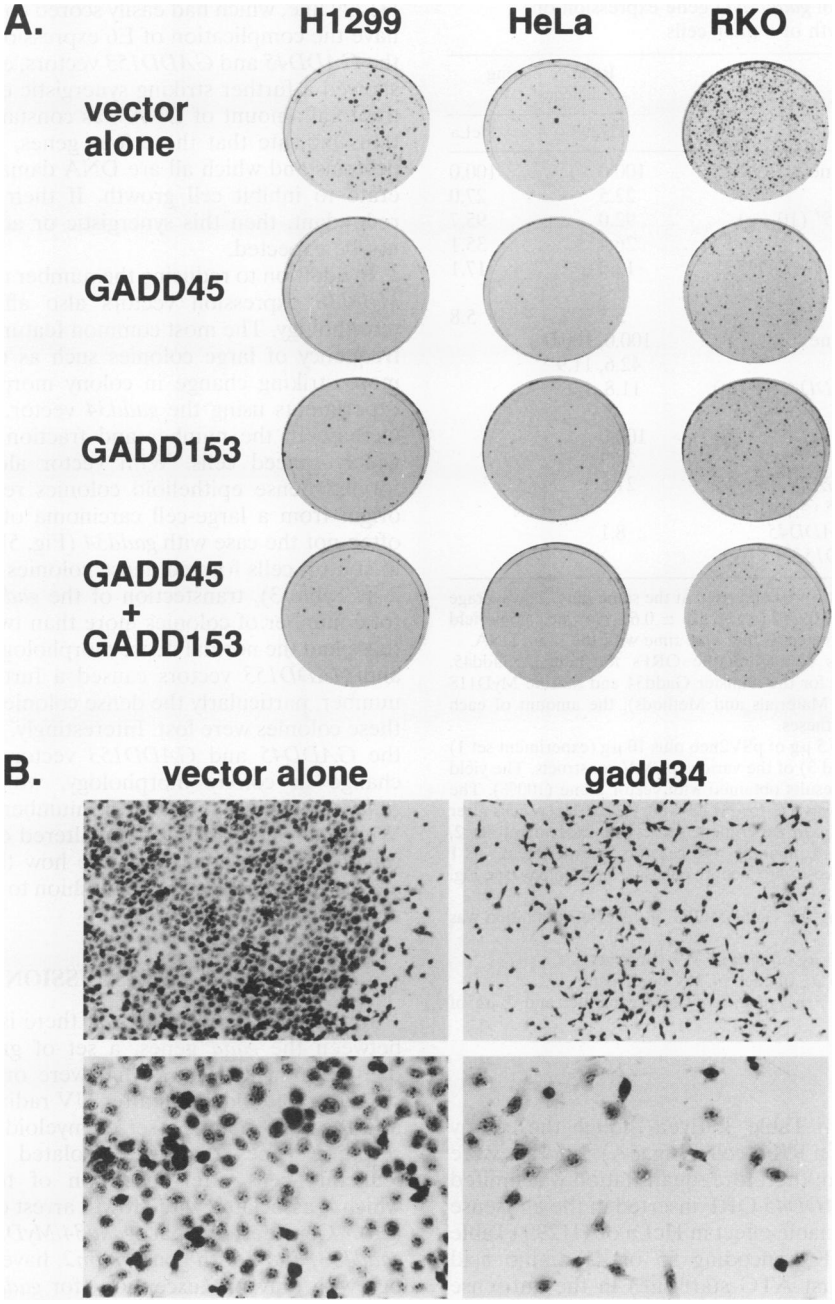

FIG. 5. Suppression of cell growth with Gadd expression vectors. (A) Human H1299, HeLa, and RKO cells were transfected with equal total amounts of pCMV.3 vector and pSV2neo as in Table 3, and G418-resistant colonies were scored 2 weeks later (see Materials and Methods). (B) Morphology of H1299 colonies transfected with pSV2neo and either pCMV.3 (vector alone) or *gadd34* expression vector. Higher magnification is shown below.

and in cells containing a viral protein, E6, that interferes with p53 function (HeLa) (47). As shown in Fig. 5A for all three lines, the yield of colonies transfected with *GADD45* or *GADD153* expression vector was clearly lower than in cells transfected with an equal amount of pCMV.3 vector. Interestingly, the combination of *GADD45* and *GADD153* vectors reduced the number of the colonies more than either expression vector alone even though the total amount of DNA transfected was the same. As seen in Fig. 5A, the transfection efficiency varied between the three cell types, but it should be noted that it remained relatively constant for each line, particularly in the same set of experiments done on the same day (data not shown). Growth inhibition was seen in all experiments and with multiple independently prepared lots of plasmid DNA.

Growth inhibition, as measured by reduced colony yield, was seen following transfection with expression vectors for the three Gadd proteins and for MyD118 (Table 3). Both *GADD45* and *GADD153* vectors reduced the yield of colonies approximately three- to fourfold in H1299 and HeLa cells in

TABLE 3. Effect of *gadd/MyD* gene expression on growth of human cells

Expt set[a]	pCMV construct[b]	Relative plating efficiency[c] (%)	
		H1299	HeLa
1	Vector (10 μg) alone	100.0	100.0
	GADD45 (10 μg)	22.5	27.0
	Antisense *GADD45*[d] (10 μg)	92.0	95.7
	GADD153 (10 μg)	26.4	35.1
	GADD45 (5 μg) + *GADD153*[e] (5 μg)	13.0	17.1
	p53 (10 μg)	3.5	5.8
2	Vector (15 μg) alone	100.0, 100.0	
	gadd34 (15 μg)	42.6, 11.9	
	gadd34 (5 μg), *GADD45* (5 μg), + GADD153[f] (5 μg)	11.8, 1.7	
3	Vector alone	100.0	
	MyD118 (15 μg)	24.3	
	GADD45 (5 μg), *GADD153* (5 μg), + vector[g] (5 μg)	21.6	
	MyD118 (5 μg), *GADD45* (5 μg), + *GADD153*[f] (5 μg)	8.1	

[a] Transfections within each set were conducted at the same time. The average variability for all experiments was 16.5% (e.g., 3.5% ± 0.6%) for the colony yield of replicate plates of cells transfected at the same time with the same DNA.

[b] pCMV.3 expression vectors containing the ORFs for human Gadd45, Gadd153, and p53 proteins and for the hamster Gadd34 and murine MyD118 proteins were constructed (see Materials and Methods); the amount of each vector used is included in parentheses.

[c] Cells were transfected with 0.5 μg of pSV2neo plus 10 μg (experiment set 1) and 15 μg (experiment sets 2 and 3) of the various pCMV constructs. The yield of colonies was normalized to results obtained with vector alone (100%). The actual number of total colonies was 372 for H1299 and 111 for HeLa cells after transfection with pCMV.3 vector in experiment set 1. For experiment set 2, scores are shown for all colonies containing >50 cells (as in experiment sets 1 and 3) (first value) and for only those with a dense cohesive morphology (see Fig. 4B) (second value).

[d] pCMV.3 construct containing the Gadd45 ORF in reverse orientation was transfected.

[e] Cells were transfected with 5 μg of each pCMV construct.

[f] Cells were transfected with 5 μg of each pCMV construct.

[g] Cells were transfected with 5 μg of each pCMV construct and 5 μg of pCMV.3 vector.

the experiments shown in Table 3. Even though the colony yield was clearly reduced in RKO cells (Fig. 5A), colonies were small and loosely attached; therefore, quantitation was limited to the other lines. The *GADD45* ORF inserted in the antisense orientation had no appreciable effect in HeLa or H1299 (Table 3). Since the small ORFs (encoding 55 or 41 amino acid proteins, based on the first ATG start site) in the antisense vector were ineffective, this result indicates that growth inhibition required expression of specific proteins. Also, it is noteworthy that the combination of *GADD45* and *GADD153* vectors reduced colony yields to levels approaching that produced by p53 in the same vector (experiment set 1 in Table 3). This synergistic effect occurred even though only half as much of each *GADD* expression plasmid was used in the sample employing both *GADD45* and *GADD153* of experiment set 1; this was done so that the amount of transfected DNA was constant in all samples of each set. Since these two *gadd* genes are expressed and stress induced in all mammalian cells examined to date, this synergistic growth inhibition probably has physiologic relevance in cellular growth control. Like the *GADD45* and *GADD153* expression vectors, *gadd34* and *MyD118* constructs both produced appreciable growth inhibition when used individually in different experiments (experiment sets 2 and 3 in Table 3); these experiments used only the H1299 line, which had easily scored colonies and which did not have the complication of E6 expression. When combined with the *GADD45* and *GADD153* vectors, either *gadd34* or *MyD118* showed a further striking synergistic effect even though again the total amount of DNA was constant. Thus, these observations indicate that these four genes, which all encode acidic proteins and which all are DNA damage inducible, can cooperate to inhibit cell growth. If their functions were strictly redundant, then this synergistic or additive-like effect would not be expected.

In addition to reducing the number of colonies, the *gadd* and *MyD118* expression vectors also affected colony size and morphology. The most common feature was a reduction in the frequency of large colonies such as observed in Fig. 5A. A more striking change in colony morphology was seen in all experiments using the *gadd34* vector, in which there was an increase in the number and fraction of colonies containing widely spaced cells. With vector alone, most H1299 cells formed dense epithelioid colonies reminiscent of this line's origin from a large-cell carcinoma of the lung, but this was often not the case with *gadd34* (Fig. 5B). While most colonies in control cells formed dense colonies (87% in experiment set 2 in Table 3), transfection of the *gadd34* vector reduced the total number of colonies more than twofold, and only 24% of these had the normal dense morphology. Addition of *GADD45* and *GADD153* vectors caused a further decrease in colony number, particularly the dense colonies, such that nearly all of these colonies were lost. Interestingly, the combination of only the *GADD45* and *GADD153* vectors did not cause such a change in colony morphology, which was dependent on *gadd34*, but only reduced the number (and size) of colonies. Whatever the reason for the altered colony morphology with *gadd34*, these studies illustrate how the different *gadd* genes also can act in an synergistic fashion to reduce the formation of normal colonies.

DISCUSSION

In this work it is shown that there is a considerable overlap between the *gadd* genes, a set of growth arrest and DNA damage-inducible genes that were originally isolated on the basis of rapid induction after UV radiation in CHO cells (15), and the *MyD* genes, a set of myeloid differentiation primary response genes that were isolated from M1 myeloblastic leukemia cells after induction of terminal differentiation, which is associated with growth arrest culminating in apoptosis (26). Considering that *gadd34/MyD116*, *gadd45*, *MyD118*, *gadd153*, and a fifth gene, *mdm2*, have no appreciable homology with only one exception (for *gadd45* and *MyD118*), it is remarkable that they have multiple common properties such as roles in growth control, unusual charge characteristics for their encoded proteins, and similarities in expression and regulation. They are distinguished from other growth arrest genes, such as the *gas* genes, *mov10*, and the prohibitin gene, in that they are DNA damage inducible. As is the case for the many genes and responses associated with growth stimulation, there are certainly multiple growth-inhibitory processes. As judged from their patterns of expression, the *gadd* genes are associated more with certain stressful growth-inhibitory responses rather than programmed responses such as occur during contact inhibition for the *gas* genes and in adult nongrowing tissues as is the case for the prohibition gene. Their similar patterns of expression and cumulative effects on growth inhibition suggest that they may participate in a variety of growth control responses.

Several lines of evidence indicate the *gadd* genes and

MyD118 may participate in effecting a variety of growth arrest responses. In the case of our results with M1 cells, the complex regulation of the four *gadd/MyD* genes suggests one or more roles for these genes in growth arrest and apoptosis, either coupled with or uncoupled from terminal differentiation. The *MyD* genes were induced early after myeloid differentiation signals, LUCM or IL-6, while the *gadd* genes were induced late near the time of apoptosis. In contrast, MMS induced apoptosis much earlier without terminal differentiation and more strongly induced the *gadd* genes than *MyD118*. Interestingly, *gadd34/MyD116* had dual responses. From these correlations in M1 cells, one could argue that the *MyD* response deals primarily with triggering the differentiation process, while the *gadd* response involves primarily apoptosis. However, the *gadd* genes clearly have roles in processes other than apoptosis, since they can be induced by treatments that do not substantially reduce cell viability, such as medium depletion or, to a limited extent, low doses of DNA-damaging agents. In addition, genotoxic stress elicits a clear apoptotic response in only certain cells and tissues (28). In a cell, growth arrest responses, such as the activation of various checkpoints, differentiation, apoptosis, and cell necrosis, probably are determined by a complex interaction involving various genes, like the *gadd* genes and *MyD*, the magnitude of their induction, and the cellular background in which they are induced.

The results in Table 3 support a role for the *gadd/MyD* genes in growth inhibition. While expression of an individual gene only had a limited effect, the combination of these various genes produced results approaching that of p53 in the same vector. This synergistic effect was not due to increased amounts of expressed protein because the amount of transfected DNA was kept constant in each experiment of Table 3. A synergistic or additive effect on growth suppression would have not been found if the different Gadd/MyD proteins had strictly duplicative or identical roles. If growth suppression were due to a nonspecific inhibition by the expression of recombinant protein, then the synergistic effect also would not occur. Another argument against a nonspecific effect of acidic proteins is the recent finding that a pCMV *MDM2* expression vector did not suppress cell growth (7). In this study, *MDM2* expression had no effect on cell growth but did block p53 action. As seen in Table 1, this protein is similar in net charge percent to the Gadd/MyD proteins. In the case of Gadd153, microinjection of this protein or a pCMV expression vector for this protein has been found recently to suppress growth, while injection of the oncogene-like breakpoint fusion protein CHOP (Gadd153)-TLS did not (3). Thus, the fusion of TLS to Gadd153 reversed its growth-suppressive effect. These and other experiments, which have not been shown, indicate that expression of the Gadd and MyD118 proteins suppresses cell growth in a synergistic manner, which is specific for these proteins and not due to a nonspecific inhibition by recombinant protein. Considering that these genes are often coordinately expressed, their proteins could very well have cooperative roles in growth inhibition.

gadd34/MyD116 has a variety of notable properties that may be relevant to its function(s). As discussed above, its response in M1 cells was biphasic, resembling responses of both the *gadd* and *MyD* genes. Its protein is more than three times larger than those encoded by the other *gadd* genes or *MyD118* and has a very high net negative charge of more than −50 in both hamster and mouse. As reported previously (29), *MyD116* has significant homology with the herpes simplex virus protein $\gamma_1$34.5, which has been shown to be a determinant of viral virulence that precludes neuroblastoma cells from triggering shutoff of protein synthesis characteristic of neuronal programmed cell death; thus, it may temporarily increase cell survival to allow viral replication (9). In H1299 cells, its expression induced a clear change in colony morphology. This could be due to scattered cell death in G418-resistant colonies that results in a dropout of cells with less dense colonies, or it could be due to some change in the cells that affects cell cohesiveness. Since the selection process with G418 also causes cell death in cells not containing stably integrated transfected DNA, these possibilities are difficult to distinguish with certainty. However, it should be noted that a small fraction of the control cell colonies had a similar appearance, suggesting that *gadd34* expression may have enhanced some process that occurs normally in this tumor line.

The complex regulation and interplay of these genes and their acidic protein products indicate that they may very well be effectors in multiple growth arrest responses, including ones involving p53. p53 protein has a clear role in the DNA damage-inducible G_1 checkpoint and in at least one type of apoptosis (22, 28). In addition, p53 protein and p53 transcriptional activity are strongly induced in human cells by MMS or medium depletion (47), indicating a role for this transcription factor in the cellular responses elicited by these treatments. In 3T3 cells, MMS, which would be expected to elicit a relatively transient growth delay compared with medium depletion, induced *mdm2*, which functions in an apparent negative feedback loop (30, 35). As shown by EMSA, p53 activity is DNA damage inducible in the 3T3 line used in our studies. Considering that IR weakly induced *GADD153* and that induction of *GADD45* and *GADD153* was reduced in AT cells after IR or MMS treatment (36), p53 may very well have role in the regulation of multiple *gadd* genes after stresses that strongly induce p53. This finding and other data to be published elsewhere indicate that p53, while not required for the *gadd* gene response to agents like MMS and medium depletion, may contribute to this response. Thus, the growth-inhibitory properties of the *gadd* genes, and perhaps others like *MyD118*, could mediate some of the cellular effects of p53.

The unusual charge characteristic of the Gadd/MyD proteins distinguishes them from certain related proteins. In the case of proteins related to Gadd153/CHOP-10, other C/EBP proteins show strong homology with this protein, particularly in the leucine zipper and DNA-binding domains (41), but their charge characteristics are very dissimilar. For example, the net charges for other C/EBP proteins derived from the indicated data bases are +5 for rat C/EBPα (PIR data base accession number PIR2:A39429), +5 for mouse C/EBPβ (PIR2:A37 279), 0 for mouse C/EBPδ (GB RO:MMCEBPD), +18 for rat C/EBPγ (PIR3:S26300), +10 for rat Crp1 (PIR2:A37280), +4 for rat Crp2 (PIR2:B37280), and 0 for mouse Crp3 (PIR: C37280). As described earlier, Gadd34/MyD116 shows homology in a 63-residue region with the herpes simplex virus protein $\gamma_1$34.5, which has a role in altering apoptosis (29). However, the charge characteristics are again very dissimilar, with $\gamma_1$34.5 encoding a basic protein. Obviously, the acidic nature of Gadd153 is not necessary for C/EBP function nor for a $\gamma_1$34.5-like function of Gadd34/MyD116. This may indicate a second function for these Gadd/MyD proteins that requires the unusual acidic charge.

While the Gadd/MyD charge properties are uncommon, an examination of published protein sequences revealed several interesting similarities with proteins associated with growth control and stress responses. The yeast checkpoint protein, Rad1, has some similarity with Gadd45 (36); this similarity includes a high net negative charge, −28, and a net charge percent of −14.5. A recent release of the SWISS-PROT database (release 27.0) was sorted according to net charge

percent, excluding peptides less than 80 residues in length. As with the similar previous sort (21), approximately 99% of the proteins were not as acidic as the Gadd/MyD group. In the case of human proteins, Gadd45 and Gadd153 had a more negative net charge percent than any known nuclear protein; and as discussed below, these proteins have recently been found to be nuclear proteins. Interestingly, several cytoplasmic proteins had similarities with the Gadd/MyD/Mdm2 proteins in charge and expression. The Alzheimer's disease amyloid A4 protein precursor (APP) was found to have a net charge percent of −8.4 in human (A4_HUMAN) and rat (A4_RAT), and −8.5 in mouse (A4_MOUSE). Interestingly, we (unpublished data) and others (2) have found that APP mRNA is increased in growth-arrested cells, including medium-depleted cells; unlike the Gadd/MyD/Mdm2 transcripts, the APP mRNA was not MMS inducible (unpublished data). The glucose-regulated proteins (GRP) have several similarities with the Gadd proteins. In particular, many contain acidic domains, and human and mouse Grp94 were found to have net charge percent values of −6.9 and −7.2, respectively. The *grp94* and *grp78* genes often show coordinate regulation (24) and have been found by multiple groups to be induced by many agents that induce the *gadd* genes (6, 17, 38). The biologic relevance of the connection between the membrane-spanning protein APP and endoplasmic reticulum proteins GRP with the Gadd/MyD group remains to be determined, but the surprisingly similar charge characteristics could suggest similar functions in different cellular compartments.

There are a variety of clues to the functions of these five acidic proteins. In addition to clear evidence for some role in stressful growth arrest responses and apoptosis (discussed above), the known properties of these proteins should assist in elucidating their specific functions. In particular, at least three, Mdm2 (34), Gadd153 (41), and Gadd45 (6a), are now known to be nuclear proteins, and two, Mdm2 and Gadd153/Chop10, are known to interact with other transcription factors. Acidic domains occur in a variety of other transcription factors, and this may indicate a possible function for these five acidic proteins in addition to their other roles (see below). In the case of Gadd153/Chop10 (41), its proposed interaction with other C/EBP proteins would not appear to require such a high negative charge, which is remarkably similar to the other Gadd/MyD/Mdm2 proteins. One possibility is that the similar net negative charges of these five proteins allow tethering to critical targets in the nucleus. Another intriguing possibility is that all five of these acidic proteins act as inhibitors to block various pathways. There is already evidence that two function in this manner. Mdm2 is known to bind to p53 and block its effects, such as after DNA damage (7), and Gadd153/Chop10 can dimerize with other C/EBP proteins to block their DNA binding (41). If all these proteins had such inhibitor functions, then their activation could have far-ranging effects by inhibiting diverse pathways.

ACKNOWLEDGMENTS

We thank Bruce Howard for providing the pCMV.3 expression vector and suggestions for its use.

We acknowledge the National Cancer Institute for allocation of computing time and staff support at the Frederick Biomedical Supercomputer Center of the Frederick Cancer Research and Development Center.

D.R. acknowledges the support of the Arthritis Foundation for his part of the project.

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