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Evidence for Distinct Kinase-Mediated Pathways in gadd Gene Responses

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ABSTRACT. We have evaluated the role of various protein kinases on the induction of the gadd (growth arrest and DNA damage inducible) genes, using a panel of protein kinase inhibitors. Our data indicate that three different stress response pathways mediating gadd gene induction are most likely regulated by different protein kinases or combinations of protein kinases. The protein kinase inhibitor staurosporine and the temperature sensitive (ts) $p34^{cdc2}$ mutant reduced induction by the alkylating agent methylmethane sulfonate (MMS) of the rodent *gadd45* and *gadd153* genes. However, staurosporine had no effect of the ionizing radiation (IR) induction of the human *GADD45*. Caffeine and 2-aminopurine, on the other hand, completely blocked this IR induction. Suramin, an antitumor drug that interferes with the interaction of growth factors with their receptors, inhibited the UV radiation induction of *GADD45* and *GADD153* but had no effect on the MMS and IR pathways. Elevated expression of *gadd45* by medium depletion (starvation) was partially reduced by the addition of either genistein or tyrphostin, two protein tyrosine kinase inhibitors, while *gadd153* was affected by tyrphostin only. Two inhibitors acting preferentially on cAMP-dependent protein kinase (PKA), *N*-[2-(methylamino)ethyl]-5-isoquinolinesulfonamide, HCl (H8) and protein kinase inhibitor (PKI), also had a moderate effect on the medium depletion-induced levels of both gadd genes. Thus, these varied effects of inhibitors on gadd gene responses point to important differences in the pathways controlling these responses. BIOCHEM PHARMACOL **55**;6:853–861, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *gadd* genes; DNA damage; kinases; ionizing radiation

The signal transduction mechanisms mediating gene induction following DNA damage are not understood completely. One emerging consensus is that DNA damage, or mechanisms leading to it, trigger a panel of different intracellular signaling events including activation of protein kinases. For example, PKC ‡ and some unidentified tyrosine kinases are activated by IR [1, 2], whereas tyrosine kinases such as Src and Raf-1 are activated by UV radiation [3]. Until recently, it was thought that the origin of signaling in the DNA damage induction of genes was nuclear. This was supported by the fact that early events occur in the nucleus where DNA absorbs UV energy or other types of "aggressions" and suffers induced lesions [4]. Moreover, in cells from patients with xeroderma pigmentosum, a UV-sensitive cancer-prone and DNA repair-deficient disorder, a much lower dose of UV is required to obtain comparable levels of enhanced transcription than in normal cells [4, 5]. Another experiment utilizing a more direct approach showed that introduction of damaged DNA into cells activated the HIV-1 long terminal repeat and that the transfer of repair enzymes into cells interferes with short-wavelength UV light (UVC)-induced HIV-1 activation [6]. However, it is becoming more and more apparent that the plasma membrane plays an early role in the activation of gene transcription following exposure to DNA-damaging agents. In HeLa cells, the UV response is initiated rapidly by the activation of membrane-associated proteins such as, as mentioned earlier, the Src-family tyrosine kinases, the small guanosine triphosphate (GTP)-binding protein (G protein) Ha-Ras [3, 7], and various growth factor receptors [8]. It has been shown that UVC can induce tyrosine phosphorylation of the EGF receptor 0.2 min after irradiation and that this activation can be specifically blocked by the receptor poison suramin [8]. One of the strongest arguments in favor of a membrane-activated signal transduction pathway is the demonstration [7] of normal activation of two transcription factors in enucle-

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‡ *Abbreviations:* PKC, protein kinase C; IR, ionizing radiation; CHO, Chinese hamster ovarian; GADD, growth arrest and DNA damage; MMS, methylmethane sulfonate; DDI, DNA damage inducible; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; PKI, protein kinase inhibitor; and CAT, chloramphenicol acetyltransferase.

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ated cells following exposure to UV radiation, although damage to mitochondrial DNA is another consideration. Irrespective of the exact primary site of damage or signal absorption, it is clear that protein kinases, and especially the ones that are inducible by phorbol esters (e.g. TPA), are involved in the induction of several DDI genes [1, 9].

In contrast to most DDI genes, the gadd genes are usually not induced by TPA, but protein kinases are most likely involved in their induction mechanisms since the protein kinase inhibitor H7 [1-(5-isoquinolinesulfonyl)-2-methylpiperazine, HCl] completely blocks their induction [10]. The gadd genes are a group of genes that were first isolated on the basis of a rapid induction following exposure to UV light in CHO cells [5]. Subsequently, these genes were found to be inducible by different types of DNA-damaging agents, and several other mammalian homologues, including human, were isolated [10]. The functions of the gadd gene products are still unknown, but recent evidence [11] indicates that they are most likely involved in negative growth control, perhaps after DNA damage or other metabolic events. Overexpression of Gadd34, Gadd45, or Gadd153 proteins by transfection of expression vectors leads to a decrease in colony formation [11] in short-term assays. While keeping the amount of transfected DNA constant, growth inhibition of at least 90% is obtained when the three gadd genes are transfected together. This rate of inhibition is nearly comparable to that seen by overexpressing the tumor suppressor p53 in a similar system. This additive effect suggested that the gadd genes cooperate to cause growth arrest rather than compete for similar function. Recent evidence indicates that the coordinately regulated gadd genes might indeed be involved in a distinctive cellular function(s). For example, microinjection of Gadd153, a C/EBP related protein also known as CHOP10, causes a block in progression from G_1 to S phase in NIH-3T3 cells [12]. *gadd34*, however, seems to associated with apoptosis. In addition to sharing homology with the herpes simplex virus gene $\gamma_{134.5}$, which prevents apoptosis of neuronal cells [13], *gadd34* is induced by agents causing apoptosis such as interleukin-6 [11]. *gadd45* for its part is a downstream effector gene of the tumor suppressor p53. It is the only gadd gene that is frequently inducible by IR. This induction is strictly dependent upon a wild-type genotype for p53 and usually correlates with the capacity to arrest in G_1 following radiation [14].

To investigate further the role of protein kinases in the induction of the gadd genes, a survey of different protein kinase inhibitors was performed, and inhibition of inducibility was evaluated by RNA-dot blot, RNAse protection, CAT assays, and western blot analysis. Our results indicate that different protein kinases are most likely involved in the IR, UV, and MMS pathways. The broad kinase inhibitor staurosporine had no effect of the p53-dependent induction of *gadd45* by IR but clearly blocked the p53-independent MMS induction. One particular effect of the drug suramin is the capacity to inhibit the growth factor receptor UV-activated signal transduction pathway. Our data indicate that suramin blocked induction of *gadd45* and *gadd153* by UV radiation but had no effect on the IR and MMS induction. This report indicates that the various pathways mediating the induction of the gadd genes are probably regulated by distinctive protein kinases.

MATERIALS AND METHODS

Cells and Cell Treatment

CHO cells and the human breast cancer line MCF-7 were grown and maintained in Ham's F12 medium supplemented with 10% fetal bovine serum. The human myeloid leukemia ML-1 line, the mouse mammary carcinoma cell line FM3A, and its temperature-sensitive mutant tsFT210 were grown and maintained in RPMI-1640 medium supplemented with 10 (ML-1) and 5% (FM3A and tsFT210) fetal bovine serum. When indicated, cells were incubated in the presence of the different kinase inhibitors for 45 min at 37° prior to DNA-damaging treatments. The concentration of the different inhibitors was as follows; genistein, 2.6 μM; PKI, 150 nM; okadaic acid, 100 nM; tyrphostin, 10 μM (GIBCO-BRL); staurosporine, 150 nM; H-7, 50 μM; H-8 {*N* - [2 - (methylamino)ethyl] - 5 - isoquinolinesulfonamide, HCl}, 1 and 10 μM; suramin, 0.3 mM (Calbiochem); 2-aminopurine, 10 mM; and caffeine, 2 and 4 mM (Sigma). The concentrations used were determined based on the IC_{50} provided by the manufacturers for *in vitro* assays and on concentrations previously reported [15–18] in similar *in vivo* systems. It has been shown [15–18] that under those conditions the protein kinases were blocked *in vivo* without affecting cell viability. The media were from GIBCO-BRL, and the serum was obtained from Hyclone. Following incubation, cells were exposed either to γ-ray radiation (20 Gy) or to the alkylating agent MMS (Sigma) at 100 μg/mL in the presence of kinase inhibitors. IR was performed as previously described [5, 19], except that cells were irradiated with a ^{137}Cs source at 5.5 Gy/min. Cells were harvested 1 hr after γ-irradiation or 4 hr after MMS treatments. The growth arrest condition (G_0) was performed on medium-depleted cells. Briefly, the cells were plated at 3×10^6 cells/p150 plate. The cells were refed with fresh medium each day for 2 days and then were not fed for 2 days. Experiments were performed on day 5 on about $3–5 \times 10^7$ cells/plate. The different protein kinase inhibitors were added to the medium for either 2 or 4 hr, and the cells were harvested.

Temperature-Sensitive Cells tsTF210

The mouse mammary carcinoma parent cell line FM3A and its mutant, tsFT210, were grown and maintained at the permissive temperature (32°). The tsFT210 mutant line was transferred at the non-permissive temperature (39°), either 18 or 2 hr prior to the addition of MMS. Cells were incubated for 4 hr in the presence of MMS and harvested, and the RNA was isolated as described below.

Plasmid Clones

Plasmids pHg45CAT-1 and p5W1 containing human *GADD45* and *GADD153* promoters, respectively, upstream of the reporter gene *CAT* were described previously [20]. Stable transfectants were made in human colon carcinoma RKO cells by selection for resistance to the neomycin antibiotic (G418, GIBCO-BRL) [20].

RNA Isolation and Analysis

Cells were lysed in 4 M guanidine thiocyanate, and RNA was isolated by the acid phenol method [21]. Quantitative RNA dot-blot hybridization was carried out as described previously [22]. Briefly, four dilutions (0.4, 0.2, 0.1, and 0.05 μg) of each sample were used for each RNA analysis. For hybridization, labeled inserts of the following cDNA clones were used: pXR45m, a nearly full-length CHO *gadd45* clone; pDDIA34, a partial-length CHO *gadd34* clone; and pDDIA175, a partial-length CHO *gadd153* clone. The relative poly (A) content of each sample was estimated by using a labeled polythymidylate probe [22]. For RNase protection assays, reagents wee obtained from Ambion, Inc., and the procedure was similar to that of the manufacturer with only minor modifications. The plasmids pRibo-Hg45 and pGAPD were linearized with *Hind*III or *Bam*HI, respectively, and *in vitro* transcription was carried out at 4° for 1 hr with T3 or T7 RNA polymerase, respectively. *GADD45* and *GAPD* riboprobes were labeled with [α-^{32}P]UTP at 3000 Ci/mmol, respectively. Ten micrograms of whole-cell RNA was hybridized with both riboprobes simultaneously (in the same test tube) at 53° for 15 hr and then digested with RNase A and RNase T1. Following proteinase K digestion and phenol/chloroform extraction, the samples were analyzed on an 8 M urea/5% acrylamide gel. Protected bands were visualized by autoradiography and were quantitated with a Betascope (Betagen, Inc.). The relative level GADD45 mRNA was determined by normalizing *GADD45* counts to the *GAPD* counts in each sample.

Western Blot

Total cell lysates were obtained as follows. Cells were washed with PBS and counted, and the cell pellets were resuspended in Laemmli sample buffer [62 mM Tris (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol, 0.003% bromophenol blue] [23] at 0.1×10^6 cells/μL. After boiling, cellular proteins were separated on a 7.5% SDS-PAGE (20 μL = 2×10^6 cell equivalents loaded per lane). Then the proteins were transferred onto nitrocellulose paper with a semi-dry blotting apparatus (Bio-Rad). The nitrocellulose paper was stained with the reversible dye Ponceau-S to ensure equivalent protein loading for each sample. Following blocking with 5% milk/PBS/0.1% Tween 20, the blot was incubated for 2 hr at room temperature with the anti-p53 antibodies pAb421 and pAb1801 (Oncogene Science) diluted 1:100 each in the blocking solution. Then the blot was washed, incubated for 1 hr at room temperature with horseradish peroxidase-conjugated goat anti-mouse (Pierce), washed again, and autoradiographed utilizing enhanced chemiluminescence according to manufacturer's instructions (ECL, Amersham).

CAT Assay

Stable transfections were performed in human colon carcinoma RKO cells as described [20]. Briefly, the cells were transfected by the Ca_2PO_4 method with 10 μg of *GADD45* promoter-CAT plasmid construct (pHg45CAT-1) and 1 μg of pSVneo. Stable transfectants were isolated based on resistance to the antibiotic neomycin (G418, GIBCO-BRL, 400 μg/mL). To reduce possible spontaneous promoter activity such as that of the immediate early genes *fos* and *jun*, the cells were incubated in low-serum (0.5%) medium 1 day prior to the experiments. Cells were harvested in PBS, centrifuged, and resuspended in 0.25 M Tris, pH 7.8. Cellular extracts were obtained by three cycles of freezing and thawing, and the amount of protein was measured with Bio-Rad protein assay reagents using BSA as a standard. Prior to performing the CAT assay, the endogenous acetylase activity was inactivated by heating the samples at 65° for 10 min. Fifteen micrograms of protein was used for each assay. The assay was run overnight at 37° and performed as previously described [24]. Acetylated products were separated from substrate on TLC plates, and radioactivity was counted on a Betascope model 603 Blot analyzer (Betagen). Extracts from mock-transfected cells were used to measure background activity. After subtracting the background activity, the specific CAT activity was determined by calculating the fraction of chloramphenicol that had been acetylated. Relative CAT activity was determined by normalizing the activity of the treated sample to that of the untreated sample. CAT activity was measured over the linear range of chloramphenicol acetylation such that the fraction acetylated was proportional to added protein.

RESULTS

Inhibition of gadd45 *and* gadd153 *MMS Induction by Different Protein Kinase Inhibitors in CHO Cells and Effect on mRNA Levels Induced by Medium Depletion*

The effects of different protein kinase inhibitors on the induction of the gadd genes in CHO cells are surveyed in Table 1. As reported previously [25], we have found that the broad protein kinase inhibitor H7 inhibits completely the induction of *gadd45* and *gadd153* by MMS in CHO cells. To evaluate the effects of the different inhibitors on the already induced levels of genes, we have evaluated the effects of the various agents on growth arrested cells (G_0, medium depletion). This growth arrest condition includes depletion of many nutrients. It has been reported [26] that depletion of glucose alone can trigger *gadd153* induction, and probably the other gadd genes too, under this condition

TABLE 1. Inhibition of gadd gene induction by different protein kinase inhibitors in CHO cells

Kinase Inhibitors	Relative mRNA[a]*							
	gadd45				gadd153			
	G_0t†/2 hr	G_0/4 hr	MMS	Control‡	G_0/2 hr	G_0/4 hr	MMS	Control
No inhibitor	16.6 ± 1.9	*§	19.5 ± 2.3	1.0 ± 0.1	33.3 ± 2.4	*	15.2 ± 1.1	1.0 ± 0.4
H7	8.5 ± 1.3	6.5 ± 1.6	0.8 ± 0.1	1.7 ± 0.4	14.0 ± 2.6	9.7 ± 1.2	0.6 ± 0.4	0.5 ± 0.2
Staurosporine	ND‖	ND	6.1 ± 0.9	0.9 ± 0.2	ND	ND	5.4 ± 1.4	0.9 ± 0.3
PKI	12.6 ± 1.0	5.7 ± 0.9	15.1 ± 0.9	1.2 ± 0.2	26.3 ± 2.6	16.0 ± 1.8	10.8 ± 3.4	1.1 ± 0.6
H8	12.3 ± 2.1	8.8 ± 0.7	18.6 ± 2.8	0.9 ± 0.1	27.0 ± 3.5	19.7 ± 1.9	13.6 ± 5.5	0.9 ± 0.2
Genistein	7.3 ± 1.3	8.9 ± 1.8	25.9 ± 1.3	1.7 ± 0.1	32.0 ± 2.8	36.7 ± 3.4	33.3 ± 2.3	1.5 ± 0.4
Tyrphostin	8.6 ± 0.9	7.5 ± 0.9	14.5 ± 0.5	1.2 ± 0.4	18.6 ± 2.2	14.2 ± 2.3	15.8 ± 2.4	1.2 ± 0.5
Okadaic acid	15.5 ± 2.1	14.3 ± 2.4	17.3 ± 0.6	1.4 ± 0.6	30.0 ± 0.9	32.2 ± 3.4	20.7 ± 0.8	0.4 ± 0.2

*[a] Relative fold induction as determined by RNA quantitative dot-blot hybridization. The relative values (average of 4 dilutions) were compared with those of untreated samples in log phase and normalized to poly (T) content (Materials and Methods). Then the poly (A) RNA was extracted and analyzed as described in the text. Values are means ± SD.

† G_0 refers to growth arrested cells by medium depletion for 2 days.

‡ Control: inhibitor alone in absence of inducing agents.

§ The gadd genes are constitutively expressed under medium depletion conditions. Therefore, their levels at 2 and 4-hr are considered similar.

‖ ND-not determined.

[26]. Since the gadd genes are constitutively expressed under medium depletion, only the 2-hr time point was measured for the control sample. Our results indicate that the H7 inhibitor also affected the levels of the gadd genes induced by the growth arrest conditions but the inhibition was not as strong as the one obtained with the MSS induction. The different effect of the inhibitor on these two induction mechanisms indicates a distinct level of H7 sensitivity for the protein kinase(s) involved. The effect on the already induced levels of the genes probably indicates an effect on *de novo* RNA synthesis, since under these conditions the message stability of *gadd45* and *gadd153* genes is not increased even though they are both constitutively expressed [27]. A previous report [15] indicated that a 50 nM concentration of staurosporine, a serine/threonine protein kinase inhibitor, did not affect the induction of *gadd153* by MMS. Here we have evaluated the effect of this kinase inhibitor at a higher concentration, 150 nM, which is still a concentration that does not cause cytotoxicity after 4 hr [16]. Our data indicate that under these conditions staurosporine markedly reduced the induction of *gadd45* and *gadd153* by MMS. Two other serine/threonine protein kinase inhibitors, PKI and H8, having a preference for protein kinase A, were also investigated. The two inhibitors had only a moderate effect on the induction of both genes, with PKI having the most pronounced effect of the 4-hr time point of the growth arrest conditions. In our panel of protein kinase inhibitors, two compounds, genistein and tryphostin, have a higher specificity for protein tyrosine kinases. The protein tyrosine kinase inhibitor tryphostin affected mainly the growth arrest-induced level on *gadd153* mRNA, whereas genistein had no inhibitory effect of either the *gadd153* G_0-induced level or on the MMS induction and only a moderate inhibitory effect on the *gadd45* G_0-induced level. Okadaic acid, a protein phosphatase inhibitor, had no inhibitory effect on the medium-depleted induction of both gadd genes. This suggests that protein dephosphorylation is not involved in the glucose depletion/nutrient starvation induction pathway.

Effects of Protein Kinase Inhibitors and Caffeine on GADD45 *Induction in Human Myeloid Leukemia ML-1 Cells*

In a previous report [14], we demonstrated that the induction of *GADD45* by ionizing radiation is dependent on a wild-type (wt) genotype for the tumor suppressor p53. Since caffeine can block the induction of p53 by IR, we wanted to evaluate its effect on *GADD45* induction. To measure this activity, we used ML-1 cells, a human line having a wt p53 genotype [14, 28]. This could not have been measured in CHO cells since induction of *gadd45* by IR does not occur in these cells [5]. RNase protection analysis (Fig. 1) indicated that caffeine blocked the induction of *GADD45* by IR (Fig. 1A) but has no effect on the MMS induction (Fig. 1B). Hence, the inhibitory effect of caffeine on the IR induction of *GADD45* correlates well with its effect on p53. We also evaluated the effects of two broad protein kinase inhibitors on *GADD45* induction. The RNase protection assays (Fig. 1C) indicate that 2-aminopurine blocked *GADD45* induction by both γ-ray and MMS. In contrast to 2-aminopurine, staurosporine had no effect on the γ-ray induction, but as it was the case in CHO cells, which lack functional p53 (Table 1), the MMS induction was inhibited. None of the inhibitors tested had any significant effect on the mRNA basal levels (data not shown). The protein tyrosine kinase inhibitor genistein gave similar results in both ML-1 and CHO cells in that no inhibitory effect was detected (data not shown).

Inhibition of p53 Protein Induction

Since the tumor suppressor p53 mediates the IR induction of *GADD45* [14], we wanted to evaluate the effect of

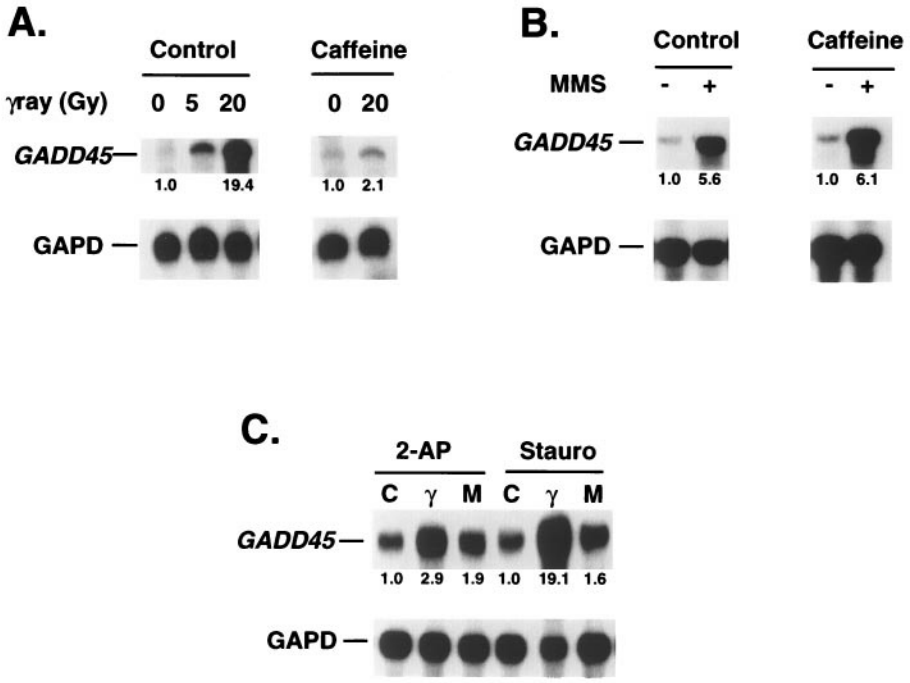


FIG. 1. Mediation of *GADD45* induction by γ-ray and MMS. The RNase protection assay was performed on ML-1 cell extracts. Cells were treated with different inhibitors 45 min prior to exposure to DNA-damaging agents. (A) Effect of caffeine (4 mM) on *GADD45* induction by γ-ray. (B) Effect of caffeine on *GADD45* induction by MMS. (C) Effect of 2-aminopurine (2-AP) and staurosporine on the γ-ray or MMS induction of GADD45 mRNA. The relative fold induction values, compared with the untreated samples ("C" no inducer), are indicated at the bottom of each *GADD45* sample. Cells were harvested 1 hr after γ-ray or 4 hr after MMS treatments.

different inhibitors on p53 induction. Induction of p53 protein was measured by western blot analysis after pretreatment of the ML-1 cells with different agents before exposure to DNA-damaging conditions. As previously reported [29], our data (Fig. 2) indicated that p53 protein is induced by MMS and γ-rays. The protein tyrosine kinase inhibitor genistein had only a mild effect on p53 induction, whereas staurosporine and caffeine inhibited induction by both MMS and γ-ray. The effect of staurosporine was more potent on the MMS induction, which is also the case for *gadd45* induction in CHO and ML-1 cells (Table 1 and Fig. 1C). Even though staurosporine did not affect the induction of *GADD45* by IR (Fig. 1C), it partially blocked the p53 induction. This might indicate that only small amounts of p53 protein are required to mediate *GADD45* induction or that even though staurosporine seems to affect p53 stability, it might not alter its activity. Caffeine dose-dependent inhibition of p53 protein induction by IR has been reported previously and correlates here with the inhibition of *GADD45* induction (Fig. 1A). We have already reported [29] that induction of *GADD45* by MMS in not restrictive for p53. The results presented here also agree with this previous finding since induction of p53 by MMS was blocked by caffeine (Fig. 2), but *GADD45* induction was not (Fig. 1B).

Inhibition of the UV Induction Pathway by Suramin

The effect of the inhibitor suramin was evaluated on the induction of *GADD45* and *GADD153*. As shown in Fig. 3 and Table 2, suramin inhibited the induction of *GADD45* by UV radiation but had no effect on the MMS induction. The induction of CAT activity under the control of the *GADD45* promoter (pHg45CAT-1) was reduced by 80% in the presence of suramin. This effect was similar under both conditions, low (0.5%) and high (10%) serum (data not

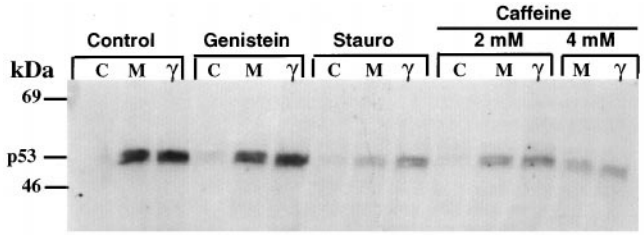


FIG. 2. Inhibition of p53 protein induction by caffeine and staurosporine. The western blot analysis of p53 was performed on ML-1 cellular extracts. ML-1 cells were treated with different inhibitors 45 min prior to exposure to DNA-damaging treatemtns. The cells were harvested 1 hr after γ-ray and 4 hr after MMS treatments and lysed in Laemmli buffer as described under Materials and Methods.

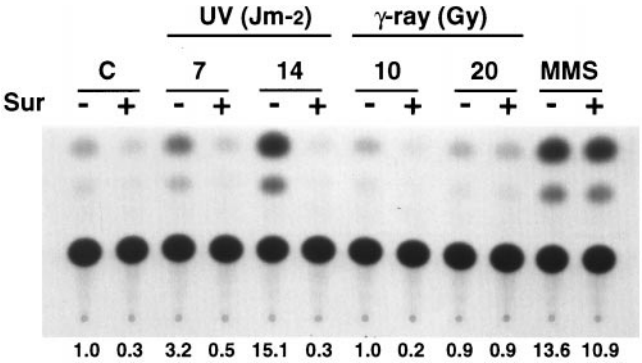


FIG. 3. Inhibition by suramin of UV-C-induction of *GADD45*. Typical results obtained from CAT assays performed on RKO cells stably transfected with a CAT reporter gene under the control of the human *GADD45* promoter (pHg45CAT-1). The cells were transferred to low serum (0.5% FCS) medium 16 hr prior to treatments. Twenty-four hours following the treatments, the cells were harvested and the protein was extracted. The relative fold induction (corrected for background) indicated below each sample was normalized to the untreated sample (control in the absence of suramin).

shown). A comparable effect was also noticed with *GADD153* (Table 2), where a 6.6-fold induction was reduced to 2.7-fold in the presence of suramin. With the exception of the two general protein kinase inhibitors, 2-aminopurine and H7, all the other protein kinase inhibitors were tested for the UV induction. None of the agents tested inhibited the UV induction as strongly as suramin in this system (data not shown). Besides suramin, genistein and H8 were the only inhibitors that showed some inhibitory effect on *GADD45* promoter; they both reduced the UV activation by less than 30%. These results indicate that the main protein kinase involved in the UV induction of the gadd genes is suramin sensitive, while other protein kinases might also be involved but to a lesser extent.

The effect of suramin on the IR induction pathway could not be detected with our CAT assay system. Our CAT assay construct contains *GADD45* promoter only while the presumed responsive element mediating *GADD45* IR induction is localized in the third intron of the gene [14]. To analyze the effect of suramin on the induction of *GADD45* by IR, we performed RNA analysis on ML-1 cells. The ML-1 cells were chosen for their low basal levels of *GADD45* and their high levels of inducibility. Dot blot analysis indicated that suramin had no inhibitory effect *GADD45* IR induction (data not shown).

TABLE 2. Relative fold induction of CAT activity for *GADD45* (pHg45CAT-1) or *GADD153* (p5W1) promoters in RKO cells

Treatment	Relative fold induction[a]*	
	GADD45	*GADD153*
UV (14 Jm^{-2})	15.8 ± 3.6	6.6 ± 0.8
UV (14 Jm^{-2}) + suramin	3.2 ± 0.8	2.7 ± 0.9
MMS	16.1 ± 3.4	5.4 ± 0.9
MMS + suramin	18.2 ± 3.3	5.1 ± 1.9

The *GADD45*- or the *GADD153*-promoter-CAT plasmid construct was stably integrated into RKO cells. The cells were transferred to low-serum medium (0.5%) for 24 hr prior to treatments. Where indicated, suramin was added 45 min before exposure to DNA-damaging agents.

*[a] CAT activity is expressed as a factor of fold induction of chloramphenicol acetylation compared with unirradiated control, and represents the average of 2 independent determinations in duplicate ($N = 4$).

TABLE 3. Induction of the gadd genes in $p34^{cdc2}$ wild-type and temperature sensitive (ts) mutant cells

Cell line/ Temp.	Treatment	Relative mRNA[a]*		
		gadd45	gadd34	gadd153
FM3A/ 39°	MMS	12.4 ± 0.9	6.0 ± 0.6	15.9 ± 6.5
tsFT210/ 39° (2 hr)	MMS	12.1 ± 1.1	10.7 ± 1.6	22.1 ± 5.6
tsFT210/ 39° (18 hr)	MMS	3.8 ± 0.5	4.8 ± 0.3	5.0 ± 1.4
tsFT210/ 32°	MMS	9.6 ± 0.9	7.2 ± 0.7	15.4 ± 3.4

The mouse mammary carcinoma parent cell line FM3A and its mutant tsFT210, were maintained at the permissive temperature (32°) and transferred to the non-permissive temperature (39°) for the indicated period of time prior to MMS treatment. Then the cells were exposed to 100 μg/mL of MMS for 4 hr, and the mRNA was extracted and analyzed as described in the text.

*[a] Relative values (average of 4 dilutions) compared with untreated samples as determined by quantitative dot-blot assay in growing cells (Materials and Methods).

Effect of p34 Inactivation on the Induction of gadd Genes

Induction of three gadd genes by the alkylating agent MMS was investigated in the ts mutant cell line, tsFT210 (Table 3). Mutations in the *cdc2* gene cause the p34 protein kinase to become inactivated and degraded at the restrictive temperature. Our data indicate that all three gadd genes were induced in the ts cells at the permissive temperature (32°). When the ts cells were transferred 2 hr prior to MMS treatment to the non-permissive temperature (39°), induction of the gadd genes by MMS was no inhibited. However, when the ts cells were transferred at 39°, 18 hr prior to treatment, induction of all three gadd genes was reduced markedly. The reason for this difference is not clear, but one possible explanation would be that 2 hr at the restrictive temperature is not sufficient to completely inactivate p34 and might not allow enough time for most of the cells to accumulate in G_2. These data suggest that inactivation of p34 is involved in the MMS induction; however, we cannot rule out the possibility that other events related to the accumulation in G_2 are also involved. Our results also indicate that the higher temperature *per se* was not responsible for the inhibition of the gene induction since transferring the parent cell line (FM3A) at the restrictive temperature did not prevent the induction of the gadd genes.

DISCUSSION

Induction of the gadd genes following DNA damage has been reported in virtually all mammalian systems studied

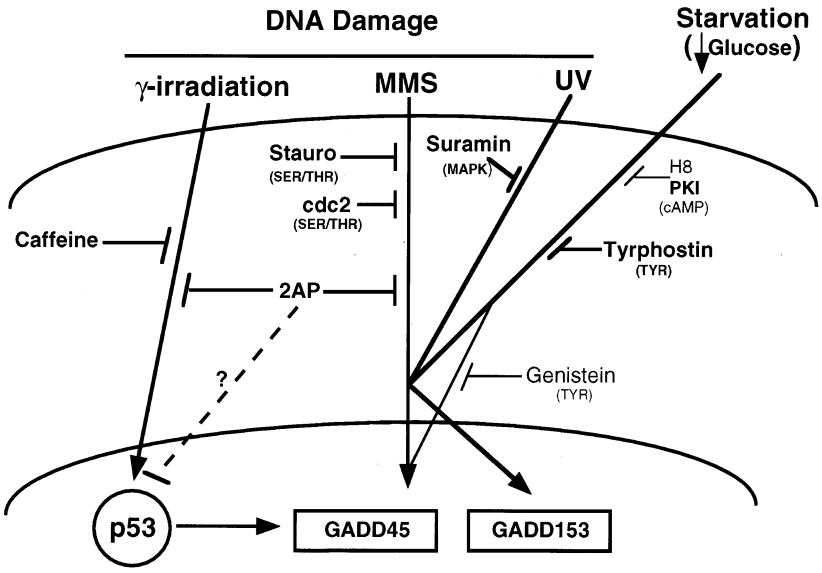
thus far. Their induction appears to be often coordinately regulated [30], and their function, though still unclear, has been associated with growth arrest [11, 14]. The mechanisms mediating the induction of gadd genes following DNA damage are poorly understood, but a previous study [25] indicated that the protein kinase inhibitor H7 completely blocks their induction. This original study suggested that protein kinases are most likely involved in the induction but did not indicate which kinase pathways were involved. The data presented here indicate that the induction mechanism of each of the gadd genes is most likely regulated by protein kinase pathways with certain distinct features. Induction by the alkylating agent MMS is inhibited by staurosporine and by the ts p34 mutant. Staurosporine is a broad protein kinase inhibitor with some preferences for PKC. However, its effect on the gadd genes is most likely due to inhibition of another type(s) of protein kinase since we have shown previously [25] that down-regulation of PKC by TPA has no effect on the induction of gadd genes. Inhibition of MMS induction by the ts p34 mutant suggests that protein kinases of the cdc2 type might be involved in this induction mechanism. The mouse mammary tumor FT210 cells grow normally at 32° but at the restrictive temperature, 39°, they lose the $p34^{cdc2}$ kinase activity and arrest in G_2 due to a temperature-sensitive lesion in the *cdc2* gene. This might indicate that the MMS pathway is functional outside G_2 and that, without being strictly dependent upon $p34^{cdc2}$, it might require other components present only in other phases of the cell cycle. However, a direct role for $p34^{cdc2}$ is reinforced by our finding that staurosporine also reduced MMS induction of the gadd genes. Staurosporine has been shown to reduce *cdc2* expression in a PKC-independent manner [31]. This is in good agreement with our previous data showing that induction of the gadd genes is PKC independent [25].

The second induction mechanism that we have looked at is the IR pathway. Previous reports have shown that *GADD45* is the only gadd gene that is frequently inducible by IR [25] and that this induction is dependent upon a wild-type genotype for the tumor suppressor p53 [14]. Our data indicate that caffeine and 2-aminopurine block *GADD45* induction by IR. The effect of caffeine on *GADD45* was somewhat expected since caffeine is known to block p53 induction by IR [28]. Interestingly, the inhibition by the broad protein kinase inhibitor 2-aminopurine might also be mediated through p53. In fact, 2-aminopurine has been reported to have actions similar to caffeine. For example, both agents override the G_2 delay imposed by DNA damage, and both have the contrary action of retarding cycle progression after TPA treatments [32]. Since induction of *GADD45* by IR is strictly dependent up on p53, it is reasonable to speculate that, as it is the case for caffeine, 2-aminopurine inhibits *GADD45* induction by interfering with p53 induction. However, we cannot rule out the possibility that 2-aminopurine prevents *GADD45* induction via a mechanism independent of p53.

The quasi exclusive inhibitory effect of the growth factor receptor poison suramin on gadd gene induction by UV radiation indicates that suramin-sensitive protein kinases such as the ERK are possibly involved in this induction mechanism [33]. The ERK are part of the MAPK pathway and include intermediate proteins such as Ras, Raf, and the ERK phosphorylating kinase MEK [33]. Several studies have already shown that exposure of cells to genotoxic stress results in the activation of one or more MAPK pathways. The ERK and the JNK/SAPK pathways are particularly sensitive to UV exposure. However, ERK is most likely the major type of protein kinase involve in the activation of gadd genes, since suramin has been shown to largely block the activation of ERK and only partially inhibit JNK activation by UV [33]. Moreover, it has been shown that suramin treatment inhibits ERK-dependent gene activation, one of these genes being the p53 downstream effector gene $p21^{CIP1/WAF1}$ [34]. $p21^{CIP1/WAF1}$ and *GADD45* have been shown to be coordinately regulated and mediate similar p53 functions such as negative growth control [35]. Therefore, it is possible that a common regulatory mechanism(s) would control the expression of both $p21^{CIP1/WAF1}$ and *GADD45* genes. Furthermore, JNK is preferentially activated by MMS [34], and our results indicate that suramin had no effect on gadd gene MMS induction.

We have reported previously [11] that suppression of growth by contact inhibition (confluency) is not effective in inducing the gadd genes whereas starvation by medium depletion is. Interestingly, growing the cells in low serum for 24–48 hr is also ineffective [11]. It seems, therefore, that nutrients other than serum are important "sensors" for the induction of the gadd genes. In this respect, it has been shown that deprivation of glucose alone is sufficient to induce *gadd153* [26]. Since the regulation of the gadd genes is frequently coordinated, it is reasonable to assume that induction of the other gadd genes by medium depletion (starvation) is also mediated by glucose deprivation. gadd45 and gadd153 mRNA levels are constitutively expressed under the medium depletion condition, and their message stability is not increased by this treatment [27]. Hence, it seems very likely that the mRNA reduction observed with the protein kinase inhibitors is due to an effect on *de novo* RNA synthesis. Several protein kinases and particularly protein tyrosine kinase(s) seems to be involved in this induction mechanism. Whether the effects of the different protein kinases involved affect the expression of gadd genes synergistically or independently remains to be elucidated.

In Figure 4 we have summarized the effects of the different inhibitors tested. Our data indicate that each induction mechanism seems to be governed by a distinctive set of protein kinases. Without being exclusive, there is a clear tendency for a suramin-sensitive protein kinase(s) to be involved in the UV response. This protein kinase(s) might be a Ser/Thr kinase of the MAPK type since suramin has been shown to inhibit their function [34], and none of the tyrosine kinase inhibitors that we have used blocked the UV induction. There is also an apparent role for a



	MMS	G_0	γ-ray	UV
2AP	++		++	
Caffeine	-		++	
Genistein	-	+	-	-
H8	-	+	-	-
PKI	-	+	-	-
Tyrphostin	-	+	-	-
Staurosporine	++		-	-
Suramin	-		-	++

FIG. 4. Possible signal transduction events in the response of the gadd genes to genotoxic stress and medium depletion. Arrows indicate induction; blunt ended bars indicate inhibition. Induction of *GADD45* by γ-irradiation is dependent on a wild-type p53 genotype [14]. The MMS induction is probably mediated by a Ser/Thr kinase, possibly $p34^{cdc2}$ (this paper). The UV light induction of the gadd genes was blocked only by suramin (this paper), which suggests involvement of the MAPK pathway (see text). Induction of the gadd genes by depletion of the medium results from glucose deprivation [26] and is most likely mediated by a combination of protein kinases with some preferences for TYR kinases (this paper). Key: Stauro, staurosporine; SER/THR, serine-threonine; TYR, tyrosine; MAPK, MAP kinase; ++, strong inhibition; −, no effect; and + mild inhibition.

$p34^{cdc2}$ type and a staurosporine-sensitive kinase in the MMS response, both of which are predominately Ser/Thr protein kinases. The IR response revolved around p53 by being sensitive to caffeine and 2-aminopurine, but we have not identified a specific protein kinase for this pathway. The mRNA levels of the gadd genes induced by medium depletion are reduced by inhibitors of protein tyrosine kinases and, to a lesser extent, by inhibitors of cAMP protein kinase. Even though the induction pathways mediating the expression of gadd genes are apparently distinct, or at least have certain distinct components, we cannot rule out the possibility that some "cross-talk" between these different systems exists.

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