gadd45 Is Not Required for Activation of c-Jun N-terminal Kinase or p38 during Acute Stress*

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Cells respond to environmental stress with activation of c-Jun N-terminal kinase (JNK) and p38. Recent studies have implicated Gadd45 and two related proteins, MyD118/Gadd45β and CR6/Gadd45γ, as initiators of JNK/p38 signaling via their interaction with an upstream kinase MTK1. It was proposed that stress-induced expression of the Gadd45-related proteins leads to MTK1 activation and subsequent JNK/p38 activation. Using embryo fibroblasts from gadd45-null mice, we have addressed the requirement for Gadd45 in mediating JNK/p38 activation during acute stress. Comparison of JNK/p38 activities in response to methyl methanesulfonate, hydrogen peroxide, UVC irradiation, sorbitol, and anisomycin treatment of gadd45+/+ and gadd45−/− fibroblasts revealed no deficiency in JNK/p38 activation in gadd45−/− fibroblasts. In addition, in wild type cells, JNK and p38 activation significantly preceded gadd45 induction with all stresses. Examination of myd118/gadd45β and cr6/gadd45γ expression in gadd45+/+ and gadd45−/− fibroblasts revealed similar induction patterns in the two cell types, which, like gadd45 expression, was delayed relative to JNK/p38 activation. We conclude that gadd45 expression is not required for activation of JNK/p38 by environmental stresses, nor are stress-induced increases in myd118/gadd45β and cr6/gadd45γ expression necessary for kinase activation in response to such insults.

gadd45 (also referred to as gadd45α) was first described by Fornace et al. (1, 2) as showing increased expression in response to DNA-damaging agents and other stresses associated with growth arrest. Although its precise function remains unclear, Gadd45 has been implicated in a variety of growth regulatory mechanisms, including DNA replication, DNA repair, G2/M checkpoint control, and apoptosis (3–8). Indeed, Gadd45 has been shown to bind to several proteins involved in these processes, including proliferating cell nuclear antigen (4), p21Waf1/Cip1 (5), and Cdc2 (7). Two other genes with homology to gadd45 have also been described: myd118 (also referred to as gadd45β) (9), implicated in myeloid differentiation, and the recently described gadd45γ (also referred to as cr6) (10, 11).

The mitogen-activated protein kinase (MAPK) signaling cascades leading to the activation of c-Jun N-terminal kinase (JNK) and p38 play important roles in mediating cellular responses to stressful stimuli (reviewed in Refs. 12–14). JNK and p38 are phosphorylated via a group of related kinases (MAPKK), which are in turn activated by another set of kinases, designated MAPKKK. While some of the upstream kinases involved in JNK and p38 activation are selective for one or the other pathway, others function in the activation of both signaling cascades. The human MAPKKK MTK1 (its mouse homologue is referred to as MEKK4) has been implicated in the activation of both JNK and p38 (15, 16). Using a yeast two-hybrid screen to detect proteins that interact with MTK1, Takekawa and Saito (10) recently identified the Gadd45-related proteins as MTK1-binding proteins. Additional experiments demonstrated that the Gadd45-related proteins were capable of enhancing MTK1 activity in vitro and that their overexpression led to activation of JNK and p38. These findings suggest a pivotal role for the Gadd45-related proteins in regulating JNK and p38 activities and Takekawa and Saito (10) proposed that stress-induced expression of Gadd45-like proteins is important in initiating the activation of JNK and p38. One concern with this hypothesis is the apparent discordance between the kinetics of JNK and p38 activation and gadd45 induction (2, 10, 17, 18), but there are no studies in which JNK/p38 activation and gadd45 expression have been simultaneously examined.

The recent generation of gadd45-null mice (19) has provided us the opportunity to directly address its role in activating JNK and p38 during stress. The results of experiments described here, comparing kinase activities of embryo fibroblasts derived from gadd45+/+ and gadd45−/− mice, indicate that Gadd45 is not required for JNK or p38 activation during stress, nor does absence of Gadd45 result in any deficiency in JNK or p38 activity. From additional experiments, in which we have systematically compared the kinetics and magnitude of activation of JNK and p38 with those for expression of mRNAs encoding gadd45, myd118/gadd45β, and cr6/gadd45γ, we conclude that stress-induced increases in the expression of these gadd45-related genes are also not involved in the acute activation of JNK or p38 during stress.

MATERIALS AND METHODS

Cell Culture and Reagents—Primary wild type and gadd45-null mouse embryonic fibroblasts (MEF), and spontaneously immortalized MEF cell lines were kindly provided by Dr. Albert J. Fornace, Jr. (National Cancer Institute). NIH3T3, COS-7, and HeLa cells were obtained from American Type Culture Collection (Manassas, VA). The cells were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum (10% fetal calf serum for NIH3T3), penicillin, streptomycin, and 2 mM glutamine and cultured at 37 °C in an atmosphere of 5% CO2, 95% air. pCMV3, Gadd45/pCMV3, Gadd45/HA-pCEP4, pCS2MT, and Gadd45/pCS2MT were provided by Dr. Albert J. Fornace, Jr. Vectors expressing hemagglutinin-tagged JNK1 (HA-

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Gadd45 expression. gadd45

m (100 μg/ml), hydrogen peroxide (600 μM), sorbitol (0.5 M), or γ irradiation (20 grays). Endogenous JNK1 was immunoprecipitated and its activity determined by an immune complex kinase assay using GST-c-Jun (1–135) as substrate. 

Cells were plated into 60-mm dishes and transfected with the appropriate expression vectors using LipofectAMINE (Life Technologies, Inc.) following the manufacturer’s instructions. The total amount of DNA added to cells was kept constant through addition of the appropriate control plasmid DNA. Following ~30-h incubation, cells were lysed, and protein extracts were used for kinase assays or Western analysis as described below.

Immunoprecipitations and Kinase Assays—Cells were lysed in 0.5 ml of lysis buffer (20 mM Heps, pH 7.4, 2 mM EGTA, 50 mM β-glycerol phosphate, 1% Triton X-100, 10% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 1 mM Na3VO4, and 5 mM NaF). Samples containing equal amounts of protein were immunoprecipitated at 4 °C overnight with 1 μg/ml of either anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-HA antibody (Roche Molecular Biochemicals) with the addition of 30 μl of 30% slurry protein A-Sepharose. The beads were pelleted by centrifugation and washed three times each in lysis buffer and kinase assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl2, 1 mM dithiothreitol, and 0.1% Triton X-100).

JNK kinase assays were performed as described previously using GST-c-Jun as a substrate (17). Total cell lysates were immunoprecipitated at 4 °C overnight with 1 μg/ml of either anti-JNK1 (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-HA antibody, and phosphorylated HA-p38 was subsequently determined by an immune complex kinase assay or Western analysis as described below.

Quantitation of Northern blots was performed using an oligomer recognizing the 18 S rRNA (17). Radioactive signals were visualized and quantitated with a PhosphorImager.

RESULTS AND DISCUSSION

Activation of JNK and p38 in gadd45+/+ and gadd45−/− MEF—To determine whether Gadd45 was necessary for JNK activation during stress, we examined the relative activation of JNK occurring in response to a panel of stresses in primary embryo fibroblasts (MEF) obtained from wild type and gadd45−/− mice (Fig. 1A). With the exception of γ irradiation, all of the stresses examined significantly elevated JNK activity. No differences in either basal or stress-induced JNK activity were observed between gadd45+/+ and gadd45−/− MEF. These results were further confirmed with spontaneously immortalized cultures of gadd45+/+ (109T) and gadd45−/− (132T) MEF, where kinase activities were monitored over a 5-h time period (Fig. 1B). Although the kinetics of activation varied for the different stresses, in all cases JNK activation was apparent at the earliest time point examined (30 min) and occurred in the absence of detectable gadd45 expression.

Similar assessment of p38 activity also revealed no deficiency in its stress-induced activation in gadd45−/− cells (Fig. 2). In fact, in most cases, p38 activation was actually higher in the gadd45−/− cells relative to gadd45+/+ MEF. Consistent with our observation in primary MEF, we detected no significant activation of JNK or p38 in response to γ irradiation in immortalized lines of either gadd45+/+ or gadd45−/− MEF (data not shown).
gadd45 Expression and JNK/p38 Activation by Acute Stress

**Fig. 4.** mRNA expression of gadd45, myd118/gadd45β, and cr6/gadd45γ in wild type and gadd45-null cells. A, representative Northern blot showing gadd45, myd118/gadd45β, and cr6/gadd45γ mRNA expression in wild type MEF at various times following treatment with 100 μM MMS. B, relative induction of gadd45, myd118/gadd45β, and cr6/gadd45γ mRNAs in wild type and gadd45-null cells at various times following their treatment with MMS (100 μg/ml), anisomycin (30 μM), or UVC (40 J/m²).

**Fig. 5.** Gadd45 expression does not result in activation of JNK or p38. A, cells were transfected with 1 μg of HA-tagged JNK and 4 μg of either one of three different Gadd45 expression vectors or the empty control vector. Thirty h following transfection, HA-JNK was immunoprecipitated from cells using an anti-HA antibody, and kinase activity was measured using GST-e-Jun as substrate. HA-JNK protein expression was monitored by anti-HA blotting. Constitutively active mutant MEK1 (ΔMEKK1) was used as a positive control for JNK activation. HA-tagged or Myc-tagged Gadd45 protein expression is shown for NIH3T3 cells. B, transient transfections were performed as described above except that HA-tagged p38 was used rather than HA-tagged JNK, and only the Myc-tagged Gadd45 expression vector was utilized. A constitutively active mutant form of MK6 was served as a positive control for p38 activation. HA-p38 activation was assessed by Western blot analysis using an anti-phospho-p38-specific antibody. Expression of the Myc-tagged Gadd45 protein was verified by Western blot analysis using an anti-Myc antibody.

Magnitude and Kinetics of Gadd45 Expression following Stress—Gadd45 protein levels were examined in the same immortal MEF populations analyzed for JNK and p38 activities above (Fig. 3). As expected, no Gadd45 expression was evident in gadd45−/− MEF, but in keeping with previous studies in other cell types, MMS was a potent inducer of Gadd45 expression in gadd45+/− cells. Importantly, however, induction of Gadd45 was significantly delayed relative to JNK and p38 activation, as no Gadd45 expression was evident until 2 h after addition of MMS (Fig. 3A). Kinetics and relative magnitude of Gadd45 expression seen with other stresses revealed similar inconsistencies with the activation of JNK and p38 (Fig. 3, A and B); hydrogen peroxide resulted in significantly lower induction of Gadd45 protein compared with MMS treatment, and no induction of Gadd45 protein was seen following UVC irradiation. However, both treatments strongly induced JNK and p38.

Expression of myd118/gadd45β and gadd45γ/cr6 mRNAs in Wild Type and gadd45-null Cells—Although the experiments above rule out a requirement for Gadd45 in stress-induced activation of JNK and p38, they do not preclude the involvement of other Gadd45-related proteins. To investigate this possibility, we examined gadd45, myd118/gadd45β, and cr6/gadd45γ mRNA expression in gadd45+/− and gadd45−/− MEF. All three mRNAs showed low expression in wild type cells in the absence of stress, but each showed enhanced expression following MMS treatment (Fig. 4A). Importantly, none of the mRNAs were significantly increased until at least 2 h post-treatment. These findings are consistent with the kinetics for Gadd45 protein expression seen above and indicate that stress-induced expression of these genes cannot account for the early activation of JNK and p38 by MMS. Similar observations were made with several other stresses (Fig. 4B, upper panel). As reported previously (10), the three transcripts appear to be differentially affected by stresses. cr6/gadd45γ was elevated to a much lesser degree by MMS than either gadd45 or myd118/gadd45β, but showed greater induction than these with anisomycin treatment. Examination of myd118/gadd45β and gadd45γ/cr6 mRNA expression in gadd45−/− MEF revealed kinetics of induction and relative fold increases similar to those seen in the wild type cells (Fig. 4B, lower panel). However, basal expression of myd118/gadd45β was higher in gadd45-null cells than in wild type MEF. Analysis of MyD118/Gadd45β and CR6/Gadd45γ protein expression could not be performed due to the lack of suitable antibodies.
Transient Overexpression of gadd45 Does Not Result in JNK or p38 Activation—Although the studies above rule out a requirement for Gadd45 in the activation of JNK and p38, they do not exclude the possibility that high levels of Gadd45 could result in JNK or p38 activation. To address this possibility, three different Gadd45 expression vectors were utilized in cotransfection studies with HA-tagged JNK (HA-JNK). HA-JNK was immunoprecipitated from cells on the following day and assayed for kinase activity. In none of three cell lines examined, HeLa, NIH3T3, or COS-7, could we see evidence for the ability of Gadd45 to activate JNK (Fig. 5A). In contrast, overexpression of a constitutively active mutant form of MEKK1 (a known activator of JNK) (20) markedly increased JNK activity. As shown for NIH3T3 cells, expression of HA- and Myc-tagged Gadd45 was verified using anti-HA and anti-Myc antibodies, respectively.

Similar co-transfection studies were performed with the Myc-Gadd45 expression vector and HA-tagged p38, after which, activation of HA-p38 was assessed by Western analysis using a phosphospecific antibody. No increase in phosphorylated p38 was evident with Gadd45 overexpression, but phosphorylation was evident with overexpression of an active mutant form of MKK6, a known p38 activator (21) (Fig. 5B). NIH3T3 cells contained a considerable amount of phosphorylated p38 even in the absence of stress, but this did not increase with Gadd45 overexpression. We have obtained similar results in several experiments using varying amounts of expression vector DNA. These findings contrast with those reported by Takekawa and Saito (10), in which overexpression of all three Gadd45-related proteins led to increased JNK and p38 activities. However, Gadd45 was the least effective of the Gadd45-related proteins in activating the kinases.

General Discussion—The recent finding that Gadd45 and the Gadd45-related proteins, MyD118/Gadd45β and CR6/Gadd45γ, can bind directly to MTK1 and enhance its kinase activity has implicated these proteins in the early signaling events leading to JNK and p38 activation. In particular, it was proposed that DNA damage and other environmental stresses result in the induction of Gadd45-related proteins, which in turn activate MTK1 leading to activation of JNK and p38. Our observation that JNK and p38 activation in response to environmental stresses clearly precedes induction of all three gadd45-related genes argues strongly against the hypothesis that stress-induced levels of the Gadd45-related proteins are involved in the acute activation of JNK and p38. However, it remains possible that stress-elevated levels of the Gadd45-related proteins could contribute to the activation of JNK or p38 in other stress paradigms in which there is a later onset and sustained activation. Such a scenario was suggested in a recent report where BRCA1 overexpression was found to result in both induction of GADD45 and JNK-dependent apoptosis, but these effects have not been functionally linked (22). It is also possible that the Gadd45-related proteins serve a role in regulating MTK1 activity that can be satisfied by basal levels of the proteins, but that MTK1 activation in cells requires some additional factor(s) that is influenced by stress. It is important to emphasize that while MTK1 is localized within the cytoplasm, Gadd45 is present mostly in the nucleus and has been implicated in a variety of nuclear-associated processes (e.g. replication, DNA repair, and cell cycle arrest). Its enhanced expression during stress is likely related to these important functions.

Finally, our studies with gadd45-null MEF demonstrate that at least this member of the Gadd45-related gene group is not required for acute activation of JNK or p38. However, our findings do not exclude the possibility that a function for Gadd45 in regulating JNK/p38 signaling could be compensated for by basal expression of Gadd45β and/or Gadd45γ in the gadd45-null MEF. Even in such a case, stress-induced increases in these proteins are clearly not necessary for the acute activation of JNK and p38 following stress. Obviously, further studies, including targeted disruption of the other Gadd45-related genes will be necessary to address whether any of the Gadd45-related proteins serve an obligatory function in regulating the activities of these stress-activated kinases.

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