Communication

Stress-induced JNK Activation Is Independent of Gadd45 Induction*

(Received for publication, July 14, 1999, and in revised form, August 5, 1999)

Eitan Shaulian and Michael Karin†‡
From the Laboratory of Gene Regulation and Signal Transduction, Department of Pharmacology, School of Medicine, University of California, San Diego, La Jolla, California 92093-0636.

DNA damage and environmental stress activate signaling and induce genes involved in cell cycle and cell death. Expression of the Gadd45 protein is induced following DNA damage and other stress. Gadd45 is believed to play a role in growth arrest and possibly in cell death. The JNK signaling pathway is also activated by some DNA-damaging agents. This activation leads to phosphorylation and activation of transcription factors, such as c-Jun/AP-1 and ATF2, which mediate immediate early gene induction. Recently Gadd45 was suggested to be involved in JNK activation. However, as this suggestion relied on in vitro experiments and ectopic overexpression of Gadd45 protein, we examined whether physiological levels of Gadd45 that are induced following exposure to DNA damaging agents and stress can lead to JNK induction. We found that JNK activation by UV irradiation and anisomycin treatment precedes the induction of gadd45 mRNA by these agents. Gadd45 protein induction by methyl methanesulfonate also lagged behind JNK activation. The use of protein synthesis inhibitors suggested that newly synthesized proteins, including the stress-induced Gadd45, make only a marginal contribution to JNK activation. We also found that stresses such as γ irradiation induce Gadd45 and do not activate JNK in mouse fibroblasts. Therefore, stress-induced JNK does not depend on Gadd45 induction.

Exposure of cells to environmental stress results in activation of several signal transduction pathways. A major factor in the cellular response to DNA damage and other types of stress, including nucleotide pool depletion and hypoxia, is the p53 tumor suppressor gene (1, 2). Upon detection of DNA damage the p53 protein, which functions as a transcription factor, is stabilized, and its elevated level results in induction of p53 target genes (3). GADD45 is one of several known p53 target genes. However, while GADD45 induction by γ radiation is p53-dependent (4), other factors such as short wavelength ultraviolet (UV) light, methyl methanesulfonate (MMS), prostaglandin J2, and camptothecin can induce Gadd45 in the absence of active p53 (5–7). Its rapid induction following exposure to DNA-damaging agents suggests that Gadd45 has a role in the response to DNA damage and other cellular insults. Several biological activities have so far been suggested for Gadd45, including involvement in DNA repair. Reduced Gadd45 expression correlates with decreased DNA repair (8). The ability of Gadd45 to modify DNA accessibility on damaged chromatin (9) and to interact with PCNA (10), a protein involved in DNA replication and repair, might support a role in this activity. Gadd45 was also shown to be an essential component of the G1/M cell cycle checkpoint induced by UV light or MMS (11). This activity is at least partially dependent on its ability to inhibit Cdc2/cyclin B kinase activity (11, 12). Gadd45 has also been implicated in apoptosis, but this biological activity is controversial, as studies suggesting that Gadd45 can induce apoptosis (13) are opposed by others, which suggest that Gadd45 induction has a protective function (8, 14).

Other pathways that are activated by various genotoxic stresses lead to activation of various MAP kinases (MAPKs), most notably JNKs and p38 (15). JNK is rapidly activated by exposure of cells to certain DNA damaging agents, such as UV light and MMS, as well as by treatment with proinflammatory cytokines and growth factors (15). Activated JNK phosphorylates and thereby enhances the transcriptional activity of several transcription factors, including c-JUN, ATF2, and Elk1. This results in induction of immediate early genes, whose end result is cell type-specific, in some cells leading to increased cell proliferation and in others to apoptosis (16–20). The duration of JNK activation was suggested to be the regulating factor that determines the cell fate (21). In some cases dominant negative mutants of c-Jun that can not be phosphorylated by JNK failed to mediate apoptosis activation (22).

A recent study presented data linking Gadd45 to the JNK pathway. Gadd45 and two other homologs, designated Gadd45β (also known as MyD118; Ref. 23) and Gadd45γ (also known as CR6), were identified through a two-hybrid screen as proteins that interact with MTK1/MEKK4, a MAPK kinase kinase (MAPKKK) that can activate JNK and p38 subgroups of MAP kinases (13). Interestingly, Gadd45 proteins not only interact with MEKK4 but can also stimulate its protein kinase activity in vitro. Transient overexpression of Gadd45 was reported to activate JNK and p38 in intact cells, presumably in an MEKK4-dependent manner (13).

We were interested to test whether stress induction of endogenous Gadd45α, the most abundant member of the family, can lead to JNK activation. In this work we report that JNK activation by UV light, MMS, and the protein synthesis inhibitor anisomycin is independent of Gadd45α induction. Furthermore, induction of Gadd45α expression after exposure of cells to γ irradiation is not linked to JNK activation. Others have found that Gadd45α-deficient cells do not exhibit any defect in JNK or p38 activation (36). Thus, at least Gadd45α is unlikely to function as a physiologically relevant activator of the JNK pathway.

MATERIALS AND METHODS

Cell Culture—3T3 mouse fibroblasts used were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C and in the presence of 5% CO2.

Kinase Assay—JNK immunoprecipitation and in vitro kinase assays using glutathione S-transferase-tagged amino-terminal c-Jun fragment
Stress-induced JNK Activation

**RESULTS**

Stress-induced Activation of JNK Precedes gadd45α Induction—To test whether Gadd45 induction correlates with JNK activation following exposure to cells of environmental stress, we exposed p53−/− mouse fibroblasts to short wavelength UV irradiation (UVC; 50 J/m²), the alkylating agent MMS (100 μg/ml), or to the protein synthesis inhibitor cycloheximide, and the effect on Gadd45 mRNA levels was determined by Northern blot hybridization. GAPDH mRNA served as a loading control. Gadd45α expression was determined by immunoblotting. A small amount of a lysate of HEK293 cells transiently transfected with a Gadd45α expression vector served as a positive control. JNK1 was immunoprecipitated and its activity determined by an immune complex kinase assay using glutathione S-transferase-c-Jun(1–79) as a substrate. The amount of immunoprecipitated JNK1 was determined by immunoblotting.

Gadd45α mRNA induction only (1–79) as a substrate were done as described previously (24). Anti-JNK1 antibody G151-333.8 (PharMingen) was used for immunoprecipitation.

Immunoblotting—The levels of immunoprecipitated JNK were determined by gel separating the proteins used in the kinase assay and transferring them to Immobilon-P membranes (Millipore) that were probed with polyclonal antibody for JNK1 (Santa Cruz Biotechnology). Phospho-JNK levels were determined by probing membranes containing equivalent levels of whole cell extract with anti-phospho-JNK antibodies (Promega). Gadd45α levels were determined using anti-Gadd45α polyclonal antibodies (Santa Cruz Biotechnology).

observed up to an hour after induction and then declined to the basal levels (Fig. 2). By comparison to UVC and anisomycin, MMS activates JNK with slower kinetics: peak activity was reached at 4-h posttreatment, although considerable activation was observed by 1 h (Fig. 3, –Cycloheximide). These experiments indicate that JNK activation at least by UV irradiation and anisomycin treatment actually precedes the induction of gadd45α mRNA expression.

**FIG. 1.** Activation of JNK by UV does not depend on Gadd45α induction. p53−/− and p53+/+ mouse fibroblasts were irradiated with 50 J/m² UVC in the presence or absence of cycloheximide (10 μg/ml) and harvested at the indicated time points. A, gadd45α mRNA levels were determined by Northern blotting. GAPDH mRNA served as a loading control. B, Gadd45α expression was determined by immunoblotting. A small amount of a lysate of HEK293 cells transiently transfected with a Gadd45α expression vector served as a positive control. JNK1 was immunoprecipitated and its activity determined by an immune complex kinase assay using glutathione S-transferase-c-Jun(1–79) as a substrate. The amount of immunoprecipitated JNK1 was determined by immunoblotting.

**FIG. 2.** Activation of JNK by anisomycin is Gadd45α-independent. p53−/− and p53+/+ mouse fibroblasts were treated with anisomycin (1 μg/ml) and harvested at the indicated times. A, gadd45α mRNA induction was determined by Northern blotting. GAPDH mRNA served as a loading control. B, Gadd45α expression was determined as described above, while JNK activation was determined by immunoblotting with an anti-phospho-JNK antibody (Promega).

**FIG. 3.** Activation of JNK by MMS does not depend on Gadd45α induction. p53−/− mouse fibroblasts were incubated with 100 μg/ml MMS in the presence or absence of cycloheximide (10 μg/ml) and harvested at the indicated time points. A, gadd45α mRNA levels were determined by Northern blot hybridization. GAPDH was used as a loading control. B, Gadd45α expression was determined by immunoblotting, and JNK activity was measured by an immune complex kinase assay. Recovery of JNK1 was determined by immunoblotting.
activation of JNK was drawn (13). However, in agreement with a previous report (5), Gadd45α was induced by both agents regardless of the p53 status (Fig. 1 and data not shown). Therefore, we examined the relationship between Gadd45α induction and JNK activation only in p53+/− cells. As expected from the induction kinetics of gadd45α mRNA, expression of Gadd45α protein can be detected only 4 h after UVC exposure (Fig. 1B). At this time point JNK activation has already declined. Treatment with cycloheximide completely abolished Gadd45α expression, but had no significant effect on JNK activation (except for a slight elevation of its basal level). Therefore, UV irradiation activates JNK independently of new protein synthesis, including that of Gadd45α. Furthermore, there was no change in the JNK activation kinetics that would support a role for Gadd45α in a late phase of the JNK activation response. Gadd45α protein induction by MMS is detected only 4 h after treatment, while JNK activity can be detected at 1 h after treatment (Fig. 3B). Therefore, induction of Gadd45α synthesis by MMS also lags behind JNK activation. Inhibition of Gadd45α expression by cycloheximide did not prevent JNK activation (Fig. 3B, + Cycloheximide). Interestingly, inhibition of protein synthesis rendered the cells more sensitive to MMS exposure and enhanced the extent of apoptosis (data not shown). This observation suggests that newly synthesized proteins induced by MMS-activated stress response pathway promote cell survival.

Anisomycin is a potent inducer of gadd45α and β mRNA (Fig. 2A and Ref. 13). However, at the concentration used for RNA induction, anisomycin, a potent protein synthesis inhibitor, completely blocked Gadd45α protein synthesis (Fig. 2B). On the other hand, as described previously (29), treatment with the same concentration of anisomycin resulted in efficient JNK activation in both p53+/− and p53−/− cells (Fig. 2B). As mentioned before, JNK activation preceded even the induction of gadd45α mRNA. These experiments strongly suggest that induction of Gadd45α protein is neither required for JNK activation by UVC, MMS, or anisomycin nor is it involved in prolonging the JNK activation response.

Elevated Levels of Gadd45 Do Not Increase JNK Activation—Exposure of cells to ionizing radiation (IR) results in a p53-dependent induction of Gadd45α (4). We took advantage of this response to test a second point, can DNA damage-induced Gadd45 activate JNK in intact cells? We exposed p53+/− and p53−/− cells to 20 grays of IR and examined JNK activation and its relationship to Gadd45α induction. As expected, Gadd45α expression increased after exposure to IR only in p53+/− cells (Fig. 4 and data not shown). In p53−/− cells the induction of Gadd45α by IR occurred with faster kinetics than its induction by UV or MMS (compare Fig. 4 to Figs. 1 and 3). Despite the rapid induction of Gadd45α, exposure to IR did not result in considerable JNK activation (Fig. 4). At most, the extent of JNK activation in IR exposed cells was 100-fold lower than in UV-irradiated cells. These results are consistent with an earlier report that IR is a very poor JNK activator in comparison with UVC and MMS (25).

DISCUSSION

DNA damage and environmental stress elicit diverse signaling and gene induction responses. The MAPK cascade that leads to JNK activation is regulated by a complicated network of upstream kinases and small G proteins, as well as by phosphatases (15). In view of this complexity, multilevel regulation is expected, and in fact different stimuli probably activate JNK via different upstream regulators. As demonstrated previously (26–28) UV irradiation results in a rapid JNK activation that is terminated within 1 h. These rapid kinetics are consistent with a process that does not require de novo protein synthesis. The rapid activation of JNK in UV-irradiated cells was shown to depend on two very early signaling events: clustering of cell surface receptors (30) and inhibition of membrane-associated tyrosine phosphatases (31).

Recently, however, it was suggested that induction of Gadd45α protein contributes to JNK activation in response to UV irradiation and other stress (13). This suggestion rested on two findings: a two-hybrid screen and overexpression experiments. Gadd45 proteins were found to bind to MEKK4, and recombiant Gadd45 proteins were found to activate MEKK4 (13). As MEKK4 was already known as one of many MAPKKS that can lead to JNK and p38 activation, it seemed reasonable to assume that induction of Gadd45 proteins, a common event in cells exposed to a variety of stresses, including UV radiation (13), can lead to JNK activation. Indeed, it was shown that overexpression of Gadd45 proteins can lead to JNK activation (13). However, it was not demonstrated that this process is mediated via MEKK4 or that it contributes to JNK activation in cells exposed to environmental stress. We examined the later possibility and found no correlation between the kinetics of Gadd45α induction, either at the protein or at the mRNA level, and JNK activation in response to three diverse stress stimuli: UV, MMS, and anisomycin. In all of these cases, JNK activation clearly preceded Gadd45α expression. Furthermore, inhibition of Gadd45α expression and presumably expression of most other induced proteins, including Gadd45β and Gadd45γ, did not abolish JNK activation by these three stimuli or alter the kinetics of activation. Gadd45α was also induced by a fourth stimulus, γ irradiation, which did not lead to considerable JNK activation. These results strongly rule out the possibility that Gadd45α makes an essential contribution to JNK activation, either at an early phase or at a late phase of the response. Although we cannot rule out the involvement of Gadd45β and Gadd45γ in JNK activation, by conjecture we suggest that it is unlikely that any of these proteins is a critical modulator of JNK activation, which is a rather rapid response to many forms of environmental stress. A more likely scenario is that JNK and/or p38 may contribute to Gadd45α induction. Indeed, it was shown recently that induction of Gadd45α by osmotic stress partially depends on p38 activity (32).

The ability of γ irradiation to induce Gadd45α without activating JNK shows clear dissociation between the events. Although the levels of p53 (33) and Gadd45α (this study) that are induced by γ irradiation are lower than the ones induced by UVC irradiation, the p53-dependent biological effects of the two forms of radiation on cell proliferation are similar; the doses that lead
to Gadd45α induction totally inhibited colony formation by mouse fibroblasts (data not shown).

The huge difference in the magnitude of JNK activation by the two forms of radiation suggest that JNK is not responsible for the observed inhibition of cell proliferation.

However, Gadd45α expression in primary human fibroblasts was shown to lead to growth arrest (11). The induction of Gadd45α by osmotic stress was also shown to correlate with growth arrest (32). So far there is little evidence implicating Gadd45α was shown to lead to growth arrest (11). The induction of cytoplasm (35).

mediated by JNK, which in nonstimulated cells resides in the cytoplasm (35).

Acknowledgments—We thank A. Fornace, N. Holbrook, and D. Liebermann for Gadd45 reagents and communication of unpublished results.

REFERENCES

1. Levine, A. J. (1997) Cell 88, 323–331.
2. Gottlieb, T. M., and Oren, M. (1996) Biochim. Biophys. Acta 1287, 77–102.
3. el-Deiry, W. S. (1996) Semin. Cancer Biol 8, 345–357.
4. Kastan, M. B., Zhan, Q., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J. Jr., (1992) Cell 71, 587–597.
5. Hollander, M. C., Alamo, I., Jackman, J., Wang, M. G., McBride, O. W., and Fornace, A. J., Jr. (1993) J. Biol. Chem. 268, 24385–24393.
6. Golubiewski, F., Bae, I., Fornace, A. J., Jr., and Pommier, Y. (1996) Oncol. Res. 8, 317–323.
7. Ohtani-Fujita, N., Minami, S., Mimaki, S., Dao, S., and Sakai, T. (1998) Biochem. Biophys. Res. Commun. 251, 648–652.
8. Smith, M. L., Kontny, H. U., Zhan, Q., Sreenath, A., O’Connor, P. M., and Fornace, A. J., Jr. (1996) Oncogene 13, 2255–2263.
9. Carrier, F., George, P. T., Pourquier, P., Blake, M., Kontny, H. U., Antinore, M. J., Garibaldi, M., Myers, T. G., Weinstein, J. N., Pommier, Y., and Fornace, A. J., Jr. (1999) Mol. Cell. Biol. 19, 1673–1685.
10. Smith, M. L., Chen, I. T., Zhan, Q., Bae, I., Chen, C. Y., Gilmer, T. M., Kastan, M. B., O’Connor, P. M., and Fornace, A. J., Jr. (1994) Science 266, 1376–1380.
11. Wang, X. W., Zhan, Q., Coursen, J. D., Khan, M. A., Kontny, H. U., Yu, L., Hollander, M. C., O’Connor, P. M., Fornace, A. J., Jr., and Harris, C. C. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3706–3711.
12. Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. (1999) Oncogene 18, 7485–7493.
13. Takekawa, M., and Saito, H. (1998) Cell 95, 521–530.
14. Torp, R., Su, J. H., Deng, G., and Cotman, C. W. (1998) Neurobiol. Dis. 5, 245–252.
15. Minden, A., and Karin, M. (1997) Biochim. Biophys. Acta 1333, F85–F104.
16. Smith, A., Ramos-Morales, F., Ashworth, A., and Collins, M. (1997) Curr. Biol. 7, 855–896.
17. Bost, F., McKay, R., Dean, N., and Mercola, D. (1997) J. Biol. Chem. 272, 33243–33249.
18. Yang, D. D., Kuan, C. Y., Whitmarsh, A. J., Rincon, M., Zheng, T. S., Davis, R. J., Rakic, P., and Flavell, R. A. (1997) Nature 389, 865–870.
19. Zanek, B. W., Boudreau, K., Rubie, E., Winnett, E., Tibbles, L. A., Zun, L., Kyrtakis, J., Liu, F. F., and Woodgett, J. R. (1996) Curr. Biol. 6, 606–613.
20. Sabapathy, K., Hu, Y., Kallunki, T., Schreiber, M., David, J. P., Jochum, W., Wagner, E. F., and Karin, M. (1999) Curr. Biol. 9, 116–125.
21. Chen, Y. R., Wang, X., Templeton, D., Davis, R. J., and Tan, T. H. (1996) J. Biol. Chem. 271, 31929–31936.
22. Behrens, A., Sibilia, M., and Wagner, E. F. (1999) Nat. Genet. 21, 326–329.
23. Abdollahi, A., Lord, K. A., Hoffman-Liebermann, B., and Liebermann, D. A. (1991) Oncogene 6, 165–167.
24. Minden, A., Lin, A., McMahon, M., Lange-Carter, C., Derijard, B., Davis, R. J., Johnson, G. L., and Karin, M. (1994) Science 266, 1719–1723.
25. Liu, Z. G., Baskaran, R., Lea-Chou, E. T., Wood, L. D., Chen, Y., Karin, M., and Wang, J. Y. (1996) Nature 384, 273–276.
26. Devary, Y., Gottlieb, R. A., Smeal, T., and Karin, M. (1992) Cell 71, 1081–1091.
27. Derijard, B., Hibi, M., Wu, I. H., Barrett, T., Su, B., Deng, T., Karin, M., and Davis, R. J. (1994) Cell 76, 1025–1037.
28. Hibi, M., Lin, A., Smeal, T., Minden, A., and Karin, M. (1993) Genes Dev. 7, 2135–2148.
29. Zinke, R., Cahill, M. A., Kracht, M., Sachsenhauser, C., Hipstock, R. A., and Nordestam, A. (1995) Mol. Cell. Biol. 15, 4300–4308.
30. Rosette, C., and Karin, M. (1996) Science 274, 1194–1197.
31. Knebel, A., Rahmsdorf, H. J., Ullrich, A., and Herrlich, P. (1996) Embo J 15, 5314–5325.
32. Kultz, D., Madhany, S., and Burg, M. B. (1998) J. Biol. Chem. 273, 13645–13651.
33. Lu, X., and Lane, D. P. (1993) Cell 75, 765–778.
34. Carrier, F., Smith, M. L., Bae, I., Kilpatrick, K. E., Lanzing, T. J., Chen, C. Y., Engilstein, M., Friend, S. H., Henner, W. D., Gilmer, T. M., Kastan, M. B., and Fornace, A. J. (1994) J. Biol. Chem. 269, 32672–32677.
35. Cavigelli, M., Dalli, F., Claret, F. X., and Karin, M. (1995) EMBO J 14, 5957–5964.
36. Wang, X., Gorospe, M., and Holbrook, N. J. (1999) Science 284, 271, 31929–31936.