Reprimo, a New Candidate Mediator of the p53-mediated Cell Cycle Arrest at the G2 Phase*

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A novel gene, Reprimo, in which induction in cells exposed to X-irradiation is dependent on p53 expression, has been isolated. Ectopic p53 expression results in the induction of its mRNA. Reprimo is a highly glycosylated protein and, when ectopically expressed, it is localized in the cytoplasm and induces G2 arrest of the cell cycle. In the arrested cells, both Cdc2 activity and nuclear translocation of cyclin B1 are inhibited, suggesting the involvement of Reprimo in the Cdc2/cyclin B1 regulation pathway. Thus, Reprimo may be a new member involved in the regulation of p53-dependent G2 arrest of the cell cycle.

Tumor suppressor genes function by affecting a variety of cellular mechanisms underlying growth, differentiation, and apoptosis, and many of these genes encode transcription factors that exert tumor suppressive effects by inducing their specific target genes (1, 2). Among them, p53 is the most commonly mutated gene in human cancers (3, 4). Upon exposure to DNA damage-inducing agents or other noxious stresses, the p53 protein is induced and/or activated, resulting in cell cycle arrest or apoptosis, so as to allow the cells to recover from damage or to eliminate the damaged cells (5–7). This p53 function is mediated mainly, if not entirely, through activation of its target genes, the products of which then function as the effector molecules (8, 9).

In this context, the regulation of the G2/M checkpoint of the cell cycle by p53 has been extensively studied. The target genes of p53 involved in this process include 14-3-3σ (10, 11), B99 (12), and Gadd45 (13, 14). Although ectopic expression of either 14-3-3σ or B99 can induce G2/M arrest in p53-deficient cells (10, 12), Gadd45 induces G2/M arrest only in cells expressing the wild-type p53 protein, indicating that Gadd45 cooperates with p53 or other p53-inducible gene(s) to arrest the cell cycle (15). In addition, p53 may suppress the G2/M transition by negatively regulating the expression of cyclin B1 (16). Thus, given the complex nature of the cell cycle machinery, it is likely that additional p53 target genes may exist for execution of the full p53 responses.

We have been investigating the interaction of p53 with another transcription factor, IRF-1,1 which was originally discovered as a regulator of the interferon response. In fact, IRF-1 regulates DNA damage-induced cell cycle arrest in collaboration with p53 through transcriptional activation of the p21WAF1/CIP1 gene (17). More recently, it has been shown that the loss of IRF-1 alleles dramatically exacerbates previous tumor predispositions caused by nullizygosity for p53 in the mouse (18). To identify the target genes of p53 and/or IRF-1 involved in cell cycle regulation and suppression of oncogenesis, we performed differential display screening of genes that are differentially expressed between x-ray-irradiated mouse embryonal fibroblasts (MEFs) obtained from wild-type and p53/IRF-1 double-deficient mice (18). We report here the cloning and functional characterization of a novel p53 target gene, Reprimo (for stop/repress). Our results collectively suggest that Reprimo may be a new member of the p53-induced proteins involved in the G2 arrest of the cell cycle.

**MATERIALS AND METHODS**

**Cell Culture**—All of the cell lines used in this study were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum. The generation of p53/IRF-1 double-deficient mice used in this report are described elsewhere (18). MEFs were isolated and maintained as described previously (17).

**RNA Blot Analysis**—MEFs were plated at a density of 5 × 10⁶ cells/15-cm plate on the day before harvesting the cells. Six hours prior to RNA extraction, the cells were irradiated with x-rays at a dose of 20 grays. RNAs were isolated as described previously (17). For RNA blot analysis, 5 µg of total RNAs was loaded per lane. The probes used to detect Reprimo was prepared by excising the StuI-XbaI fragment of pEF/HA-Reprimo (described below). The probe for Mdm2 was prepared by excising the StuI base pair XbaI-BsmI fragment from mdm2X2, a Mdm2 expression vector (19).

**Differential Display Cloning**—Total RNAs extracted from the MEFs of wild-type or p53/IRF-1 double-deficient mice exposed or not exposed to x-ray irradiation were used as the templates for differential display. Differential display was performed as described previously (20). The full-length sequence of Reprimo was determined by sequencing the 5′ and 3′-RACE and RT-PCR products. Human Reprimo was cloned by RT-PCR, based on the result obtained by a sequence similarity search. The cDNA sequences were then confirmed by sequencing the genomic clones. RACE was performed using the Marathon cDNA amplification kit (CLONTECH), and RT-PCR was carried out using SUPERSCRIPT II (Life Technologies, Inc.) according to the manufacturers’ protocols.

**Genomic Library Screening and FISH**—Genomic library screening was conducted as described previously (21). Each phage clone from the mouse and human library containing the Reprimo gene was digested with XhoI and subcloned in pBluescript. FISH was performed as described previously (22). The probe used for FISH was the 7.7-kb fragment of human Reprimo subcloned in pBluescript containing the entire coding region of the human Reprimo gene.

**Adenovirus-mediated Gene Transfer**—The Reprimo coding sequence was amplified by PCR using primers containing the StuI and XbaI sites with the p53 or other p53-inducible gene(s) to arrest the cell cycle (15). In addition, p53 may suppress the G2/M transition by negatively regulating the expression of cyclin B1 (16). Thus, given the complex nature of the cell cycle machinery, it is likely that additional p53 target genes may exist for execution of the full p53 responses.

The abbreviations used are: IRF-1, interferon regulatory factor-1; MEF, mouse embryonal fibroblast; RACE, rapid amplification of cDNA ends with polymerase chain reaction; RT-PCR, reverse transcriptase-PCR; FISH, fluorescence in situ hybridization; HA, influenza virus hemagglutinin; PI, propidium iodide; DAPI, 4,6-diamidino-2-phenylindole; kb, kilobase.
Infection was done at the multiplicity infection of 100.

The recombinant adenovirus was constructed as described previously (24) using the Takara blunting kit, and the fragment was inserted into the Sau I site of the adenovirus expression vector. The recombinant adenovirus was excised, both ends were blunted using the Takara blunting kit, and the recombinant adenoviruses expressing p53 was described previously (25).

The recombinant adenovirus expressing p53 was described previously (25). To construct the HA-tagged Reprimo adenovirus expression vector, the SauI-SalI fragment of the pEF/Ha-Reprimo expression vector was excised, both ends were blunted using the Takara blunting kit, and the fragment was inserted into the Sma I site of the adenovirus expression vector. The recombinant adenovirus was excised as described previously (24) using the Takara adenovirus expression vector kit. The recombinant adenovirus expressing p53 was described previously (25). Infection was done at the multiplicity infection of 100.

Cell Cycle Analysis—Trypsinized cells were harvested and washed once with phosphate-buffered saline. The cells were then fixed with 70% ethanol, washed with phosphate-buffered saline, and stained with PI after RNase treatment. Flow cytometric analysis was performed using a FACSCALIBUR (Beckton Dickinson).

Cell Synchronization—Cell synchronization was carried out basically as described previously (26). HeLa cells were synchronized by double thymidine block, and adenovirus infection was performed between the first and second thymidine block. After overnight incubation with 2 mM thymidine, the cells were washed with fresh medium without thymidine and released for 6 h. Then, the cells were infected with the appropriate adenovirus for 3 h. Subsequently, the cells were subjected to the second block with 2 mM thymidine for 14 h. Etoposide-induced cell cycle arrest was carried out as described previously (26). HeLa cells were released from the double thymidine block for 6 h and then treated with etoposide (20 μg/ml) for 6 h. Colcemid-induced cell cycle arrest was carried out as described above except using colcemid instead of etoposide.

Western Blotting, Glycopeptidase F Treatment, and Immunofluorescent Staining—Cells were lysed and subjected to Western blotting as described previously (27). Antibodies used in this study were anti-HA antibody (Roche Diagnostics, clone 12CA5), anti-cyclin B1 antibody (Santa Cruz Biotechnology, sc-245), anti-Cdc2 antibody (Santa Cruz Biotechnology, sc-54) and anti-phospho-Cdc2 (Tyr-15) antibody (New England Biolabs). Anti-Reprimo antibody was obtained by immunizing rabbits with synthetic oligopeptides corresponding to amino acids 90–22628 at the 5′ end of the coding sequence. PCR products were first excised, both ends were blunted using the Takara blunting kit, and the fragment was inserted into the Sau I site of the adenovirus expression vector.

RESULTS AND DISCUSSION

Identification of the cDNA Encoding a Novel Protein, Reprimo—To identify genes that are induced by p53 and/or IRF-1 following x-ray irradiation, we employed a differential display screening approach (20, 30). By screening 240 sets of primers, we identified five and two genes, for which induction was dependent on p53 and IRF-1, respectively. Two of the p53-inducible genes were Cyclin G and PAG608, which have already been reported to be p53 target genes (31, 32). The other three p53-dependent genes have not been described thus far, and cDNA expression studies have revealed that only one of them, termed Reprimo, is involved in cell cycle regulation.

As shown in Fig. 1A, Reprimo mRNA (1.5 kb) is induced following x-ray irradiation in wild-type MEFs (approximately 5-fold induction). However, in MEFs from p53-deficient mice, its basal mRNA expression level is markedly decreased, and its expression is not induced following x-ray irradiation (Fig. 1A).

In the wild-type MEFs, the kinetics of Reprimo mRNA induction is similar to that of p53-regulated Mdm2 mRNA induction (33), both culminating 3 h after the irradiation (Fig. 1B). As in MEFs, the Reprimo mRNA was found to be expressed at low levels in a variety of tissues by RNA blotting analysis using mouse multiple-tissue poly(A) RNAs (data not shown).

Sequencing analysis revealed that mouse Reprimo cDNA contains a 327-base pair open reading frame encoding a protein of 109 amino acids (Fig. 1C). The sequence around the putative ATG initiator is in good agreement with the consensus sequence for translation initiation (34), and it is preceded by two in-frame stop codons (data not shown). The predicted Reprimo protein sequence exhibits no significant homology to any known proteins using BLAST (basic local alignment search tool) sequence similarity search. Moreover, we could not find any other open reading frame encoding a known protein in this cDNA using the same method. We also cloned the human orthologue of Reprimo cDNA, which also encodes a protein of 109 amino acids, showing 98% identity with its mouse counterpart (Fig. 1C).

Reprimo Gene Induction by p53 and Its Chromosomal Localization—In view of the results presented in Fig. 1, A and B, that the DNA damage-induced Reprimo mRNA expression is observed in wild-type but not p53-deficient MEFs, we isolated the mouse Reprimo gene, which is present as a single copy (2). A 6-kb mouse genomic DNA fragment, which included the Reprimo coding sequence as well as a 3.7-kb upstream sequence, was obtained by genomic library screening. Analysis of this
gene segment has revealed that the gene does not contain introns. Although we examined the promoter region spanning up to 1 kb from the putative transcription initiation site, no definitive evidence has been obtained thus far regarding the p53 response elements in this region. Therefore, further work is required to clarify whether this promoter is activated directly or indirectly by p53. As an alternative approach to examining the inducibility of Reprimo mRNA by p53, we ectopically expressed p53 in human Saos2 cells, which otherwise lack functional p53 expression. As shown in Fig. 1D, overexpression of p53 resulted in the induction of endogenous Reprimo gene. Furthermore, such induction was also observed following x-ray irradiation of several cultured cell lines carrying wild-type p53 (data not shown). These results lend further support to the notion that Reprimo expression is regulated by p53.

In view of the finding that Reprimo is regulated by p53, it was interesting to examine the chromosomal localization of this gene. As shown in Fig. 1E, human Reprimo is mapped to 2q23 by fluorescence in situ hybridization (FISH). It is interesting that the human Reprimo gene is mapped to a locus frequently lost in lung cancer cells and neuroblastomas (35, 36). Thus, the possibility of inactivation of this gene in cancers may be an interesting subject of future investigation.

Modification of the Reprimo Protein—Reprimo protein expression was detected by immunoprecipitation of [35S]methionine-labeled proteins using rabbit polyclonal anti-Reprimo antibody (Fig. 2A). The size of this protein deduced from its primary amino acid sequence is approximately 12-kDa; however, four bands (approximately 16, 21, 23, and 40 kDa) could be detected, all of which were induced following X-irradiation in wild-type MEFs but not in p53-deficient MEFs (Fig. 2A). Consistent with this finding, ectopically expressed Reprimo tagged with influenza virus hemagglutinin peptide (HA) also expressed HA-tagged Reprimo (HA-Reprimo), and were either treated or not treated with glycopeptidase F for 24 h. The Reprimo protein was detected with anti-HA antibody.

Functional Analysis of Reprimo—X-ray-irradiated MEFs cease to multiply and become arrested at the G1 or G2/M phase of the cell cycle, and this cell cycle arrest was shown to be dependent on the expression of p53 (5–7). Because Reprimo is induced following x-ray irradiation in a p53-dependent manner, we speculated that it might play an important role in cell cycle regulation. To test this possibility, we expressed Reprimo cDNA in various cells by adenovirus-mediated gene transfer. When recombinant adenovirus expressing Reprimo cDNA was infected into human colorectal cancer cell line DLD1, expression of the Reprimo protein could be detected as early as 12 h after infection, and the expression level increased for at least 18–20 h after infection (Fig. 3A). First, we checked the proliferation of the infected cells and found that cells overexpressing Reprimo did not proliferate after 24 h after the infection, whereas the number of control cells overexpressing LacZ continued to proliferate (data not shown). When cell cycle analysis was performed, cells overexpressing Reprimo showed almost complete cell cycle arrest at the DNA content of 4 N by 36 h after adenoviral infection, whereas cells overexpressing LacZ showed normal cell cycle progression indistinguishable from that of the noninfected cells (Fig. 3B). Because the cells expressing Reprimo did not show the apoptotic phenotype until 4 days after adenoviral infection, determined by trypan blue exclusion assay or TUNEL (TdT-mediated dUTP nick end labeling) assay (data not shown), Reprimo expression is considered to have a selective effect on cell cycle arrest. Furthermore, we observed that various cell lines, including human DLD1 (mutated p53), human HeLa (wild-type p53), human Lovo (wild-type p53), human MCF7 (wild-type p53), human Saos2 (p53 null) (38, 39), and mouse NIH3T3 cells, were similarly arrested at the DNA content of 4 N with the expression of Reprimo (data not shown). These data suggest that the p53 status of cells does not affect the function of Reprimo, consistent with the notion that Reprimo is a downstream mediator of p53 action.

Reprimo Affects the Activation of Cyclin B1-Cdc2 Complex—As shown by the above experiments, ectopic expression of Reprimo leads to G2/M arrest. To determine whether the cells are arrested at the G2 or the M phase, we examined the subcellular localization of cyclin B1, in which translocation into the nucleus is one of the hallmarks of cells entering the M phase. We used cell cycle-synchronized HeLa cells infected with either control or Reprimo-expressing adenovirus to monitor the cell cycle progression. Although the majority of the cells were retained at the S and G2 phases, no difference was found between the cells expressing Reprimo or LacZ (Fig. 3C). At the S phase, Reprimo- and LacZ-expressing cells both showed very low levels of cyclin B1 expression and typical interphase nuclei, whereas after they entered the G2 phase, cytoplasmic accumulation of cyclin B1 was observed. As shown in Fig. 3C, cells expressing LacZ continued to progress to the M phase, as deduced by the nuclear translocation of cyclin B1 and the chromosomal condensation. Interestingly, however, neither nuclear translocation of cyclin B1 nor chromosomal condensation was observed in the cells expressing Reprimo, indicating that cells are arrested at the G2 phase of the cell cycle.

In addition to intracellular staining, immunoblot analysis was also performed to examine the tyrosine dephosphorylation of Cdc2, another indicator of the entry of cells into the M phase. As shown in Fig. 3D, the Cdc2 protein levels remained essentially the same in the cells expressing Reprimo and in those expressing LacZ. However, the dephosphorylation of Cdc2 was not observed in the cells expressing Reprimo, suggesting the possibility that Reprimo expression results in suppression of the Cdc2 activity. Consistently, in vitro histone H1 kinase assay of Cdc2 activity revealed that this activity was signifi-
Reprimo with Cdc2 or cyclin B1 (data not shown). Hence, Reprimo may regulate the activity of the Cdc2-cyclin B1 complex by interfering with an as yet unknown G2/M checkpoint mechanism operating in the cytoplasm. This is an important subject for future studies. Generation of Reprimo-deficient mice and screening of cancer cells for Reprimo mutation are in progress and will provide answers to the role of this novel protein in cell cycle arrest and oncogenesis.

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REFERENCES
1. Hanahan, D., and Weinberg, R. A. (2000) Cell 100, 57–70
2. Hunter, T. (1997) Cell 88, 333–346
3. Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C. C. (1991) Science 253, 49–53
4. Levine, A. J., Momand, J., and Finlay, C. A. (1991) Nature 351, 453–456
5. Gottleib, T. M., and Oren, M. (1996) Biochim. Biophys. Acta 1287, 77–102
6. Levine, A. J. (1997) Cell 88, 323–331
7. Sherr, C. J. (1996) Science 274, 1672–1677
8. El-Deiry, W. S. (1998) Semin. Cancer Biol. 8, 345–357
9. Yu, J., Zhang, L., Hwang, P. M., Rago, C., Kinzler, K. W., and Vogelstein, B. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 15417–15422
10. Hermeking, H., Lengauer, C., Polyak, K., He, T. C., Zhang, L., Thiagalingam, S., Kinzler, K. W., and Vogelstein, B. (1997) Mol. Cell 1, 3–11
11. Chau, T. A., Hermeking, H., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999) Nature 401, 616–620
12. Utrera, R., Collavin, L., Lazarevic, D., Delia, D., and Schneider, C. (1998) EMBO J. 17, 5015–5025
13. Carrier, F., George, P. T., Pourquier, P., Blake, M., Kontny, H. U., Antinore, M. J., Gariboldi, M., Myers, T. G., Weinstein, J. N., Pommier, Y., and Fornace, A. J., Jr. (1999) Mol. Cell Biol. 19, 1673–1685
14. Kastan, M. B., Zhan, Q., el-Deiry, W. S., Carrier, F., Jacks, T., Walsh, W. V., Plunkett, B. S., Vogelstein, B., and Fornace, A. J., Jr. (1992) Cell 71, 587–597
15. Wang, X., Geroese, M., and Holbrook, N. J. (1999) J. Biol. Chem. 274, 29999–29800
16. Innocente, S. A., Abrahamson, J. L., Cogswell, J. P., and Lee, J. M. (1999) Oncogene 18, 1213–1220
17. Tanaka, N., Ishihara, M., Lamphier, M. S., Nozawa, H., Matsumiya, T., Mak, T. W., Aizawa, S., Tokino, T., Oren, M., and Taniguchi, T. (1996) Nature 382, 816–818
18. Nozawa, H., Oda, E., Nakao, K., Ishihara, M., Ueda, S., Yokochi, T., Ogasawara, K., Nakatsu, Y., Ishihara, M., Ohira, Y., Hikoi, K., Aizawa, S., Ishikawa, T., Katsuki, M., Muto, T., Taniguchi, T., and Tanaka, N. (1999) Genes Dev. 13, 1240–1245
19. Haupt, Y., Barkay, Y., and Oren, M. (1996) EMBO J. 15, 1956–1966
20. Ino, T., Kito, K., Adati, N., Nishida, Y., Hidaka, H., and Takahashi, Y. (1994) FEBS Lett. 351, 231–236
21. Miyamoto, M., Fujita, T., Kinuma, Y., Maruyama, M., Harada, H., Sudo, Y., Miyata, Y., and Taniguchi, T. (1988) Cell 54, 903–913
22. Inazawa, J., Ariyama, T., Abe, T., Druck, T., Ohita, M., Huebner, K., Yanagisawa, J., Reed, J. C., and Sato, T. (1996) Genomics 31, 240–242
23. Sato, M., Tanaka, N., Hata, N., Oda, E., and Taniguchi, T. (1996) FEBS Lett. 395, 112–116
24. Miyake, S., Makimura, M., Kanegae, Y., Harada, H., Sato, T., Takamori, K., Tokuda, C., and Saito, I. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1329–1332
25. Yamane, S., Tokino, T., Yasuda, M., Kaneuchi, M., Takanashi, N., Niitsu, Y., Fujinaga, K., and Yamashita, T. (1999) J. Virol. 73, 10095–10103
26. Toyoshima, F., Moriyama, T., Wada, A., Fukuda, M., and Nishida, E. (1998) EMBO J. 17, 2728–2735
27. Tanaka, N., Ishihara, M., Kitagawa, M., Harada, H., Kinuma, T., Matsuyama, T., Lamphier, M. S., Aizawa, S., Mak, T. W., and Taniguchi, T. (1994) Cell 77, 829–839
28. Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998) Cell 92, 725–734
29. Watanabe, N., Iwamura, T., Shinoda, T., and Fujita, T. (1997) EMBO J. 16, 3609–3620
30. Liang, P., and Pardee, A. B. (1992) Science 257, 967–971
31. Okamoto, K., and Beach, D. (1994) EMBO J. 13, 4816–4822
32. Israeli, D., Tessler, E., Haupt, Y., Ellekes, A., Wilder, S., Amson, R., Telerman, A., and Oren, M. (1997) EMBO J. 16, 4384–4392
33. Barkay, Y., Juven, T., Haffner, R., and Oren, M. (1990) EMBO J. 9, 3621–3627
34. Kozak, M. (1989) J. Cell Biol. 108, 229–241
35. Otsuka, T., Kohn, T., Mori, M., Noguchi, M., Hirohashi, S., and Yokota, J. (1999) Genes Chromosom. Cancer 16, 113–119
36. Takita, J., Hayashi, Y., Kohn, T., Shiozaki, M., Yamaguchi, N., Hanada, R., Yamamoto, K., and Yokota, J. (1995) Oncogene 11, 1829–1834
37. Fan, J. Q., and Lee, Y. C. (1997) J. Biol. Chem. 272, 27658–27664
38. Polyak, K., Waldman, T. H., He, T. C., Kinzler, K. W., and Vogelstein, B. (1996) Genes Dev. 10, 1495–1502
39. Zhan, Q., Fan, S., Bae, I., Guillouf, C., Liebermann, D. A., O’Connor, P. M., and Fornace, A. J., Jr. (1994) Oncogene 9, 3743–3753

FIG. 3. Overexpression of Reprimo leads to G2 arrest. A, Reprimo expression in cells infected with recombinant adenovirus possessing Reprimo. Whole cell lysates (50 μg) obtained at the indicated times (in hours) after the infection were loaded. Reprimo protein expression was detected with anti-HA antibody. B, cell cycle analysis of the cells overexpressing Reprimo. The top panel shows the cell cycle progression pattern for noninfected cells. The horizontal axis shows the DNA content. Two peaks are seen for noninfected cells, which correspond to DNA content of 2 (200) and 4 x (400). The bottom left and right panels show the results obtained for cells overexpressing LacZ and Reprimo, respectively, at the indicated times following infection with recombinant adenoviruses. C, subcellular localization of cyclin B1 and nuclear morphology of LacZ- or Reprimo-overexpressing cells. Cells were stained with cyclin B1 antibody or DAPI at the indicated times after release from the double thymidine block. The same microscopic field is shown for each staining. Cells arrested with etoposide or colcemid are shown in the bottom panels as the control for G2 and M phase-arrested cells, respectively. D, protein levels and phosphorylation levels of Cdc2 in cells overexpressing Reprimo. Whole cell lysates (50 μg) were prepared at the indicated times from LacZ- or Reprimo-overexpressing cells after release from double thymidine block and were subjected to Western blotting with anti-phospho-Cdc2 (P-cdc2) or anti-Cdc2 (cdc2) antibody. E, Reprimo is a cytoplasmic protein. HeLa cells were infected with Reprimo-expressing adenovirus and subjected to immunofluorescent staining. Ectopically expressed Reprimo was detected with anti-HA antibody.