c-Abl Neutralizes the Inhibitory Effect of Mdm2 on p53*

(Received for publication, December 21, 1998, and in revised form, January 29, 1999)

Ronit Vogt Sionov‡, Eli Moollem‡, Michael Berger‡, Anat Kazaz‡, Offer Gerlitz‡, Yimon Ben-Neria‡, Moshe Oren§, and Ygal Haupt†
From the ‡Lautenberg Center for General and Tumor Immunology, The Hebrew University Hadassah Medical School, Jerusalem 91120, Israel and the §Department of Molecular Cell Biology, The Weizmann Institute of Science, Rehovot 76100, Israel

Upon exposure to stress signals, the p53 tumor suppressor protein is stabilized and induces growth suppression. p53 activities are efficiently inhibited by the Mdm2 oncoprotein through an autoregulatory feedback loop. In addition, Mdm2 promotes p53 degradation, thereby terminating its growth inhibitory signal. Hence, p53 exerts its effects during the interval between p53 activation and the subsequent inhibition by Mdm2. Modulation of this interval by regulatory proteins may determine the extent and duration of p53 activity. Recent studies have shown that the c-Abl protein-tyrosine kinase binds p53 and enhances its transcriptional activity. Here we provide an explanation for the cooperation between these proteins. We demonstrate that c-Abl increases the expression level of the p53 protein. The enhanced expression is achieved by inhibiting Mdm2-mediated degradation of p53. This provides a likely mechanistic explanation for the findings that c-Abl overcomes the inhibitory effects of Mdm2 on p53-mediated transcriptional activation and apoptosis. These results suggest that c-Abl modulates the time window within which p53 remains active. The ability of c-Abl to neutralize the inhibitory effects of Mdm2 on p53 may be important for its growth inhibitory function.

The p53 tumor suppressor plays a key role in the cellular response to stress. The p53 protein exists in a latent inactive form with a short half-life. However, in response to stress the p53 protein undergoes post-translational modifications and accumulates (1, 2). Activation of p53 induces cell growth arrest or apoptosis. These activities are mediated by its target genes, such as p21

Importantly, Mdm2 also promotes the rapid degradation of p53 through the ubiquitin-proteasome system (4–6). Since mdm2 is a direct target gene of p53, it shuts down its own expression through a negative autoregulatory feedback loop (reviewed in Ref. 3).

The activity of p53 is also positively regulated. A physical and functional link has been demonstrated between p53 and c-Abl, a nonreceptor tyrosine kinase. Both genes are activated in response to similar genotoxic stresses (7) and undergo phosphorylation and activation by DNA-dependent protein kinase or ATM (Ref. 8; reviewed in Ref. 9). This link suggests that the two proteins may act in a common pathway during the cellular response to DNA damage. c-Abl binds p53 in vitro (10) and in vivo (11), and consequently the transcriptional activity of p53 is enhanced (10, 12). p53 is required by active c-Abl to induce cell growth arrest and apoptosis (10, 11, 13, 14).

This study focuses on the mode of cooperation between c-Abl and p53. We demonstrate that c-Abl enhances p53 activity through inhibition of Mdm2-mediated p53 degradation. As a consequence of this neutralization of Mdm2, the p53 protein is stabilized in an active form. Therefore, we suggest that by relieving the inhibitory effect of Mdm2, c-Abl can act as a positive regulator of p53.

EXPERIMENTAL PROCEDURES

Cells and Transfection Assays—Mouse embryo fibroblasts were grown in Dulbecco’s modified Eagle’s medium, H1299, and Saos-2 cells were maintained in RPMI with 10% fetal calf serum. Transfections, luciferase assay, and Western blot analysis were carried out as described previously (15). The antibodies used were: anti-human p53 monoclonal antibodies PAb1801, PAB421, and DO1, anti-human Mdm2 SMP14, anti-c-Abl monoclonal antibody (8E9), and anti-a-tubulin antibody (DM 1A Sigma).

Immunoprecipitation was carried out essentially as described previously (15). p53 complexes were immunoprecipitated with PAb421, resolved by SDS-polyacrylamide gel electrophoresis, and subjected to Western blotting using anti-Mdm2. Flow cytometric analysis was carried out essentially as described in Haupt et al. (15). Samples were analyzed in a cell sorter (FACSCalibur, Becton Dickinson).

Plasmids—Expression plasmids were: human wt p53 (pCMV-Neo-Bam-p53), mutant p53 (pRCP53Gln14,Ser19 and pRCP53Gln22,Ser23); human wt mdm2 (pCMV-Neo-Bam-mdm2) and human mutant mdm2 (pCMV-Neo-Bam-mdm2N5); mouse mdm2 (pOOC-mdm2X2; Ref. 15); and mouse wt c-abl (pCMV-c-abl IV) and kinase defective c-abl (pCMV-c-abl K290R). All of these cDNAs were driven under the CMV promoter. The reporter plasmids used were: cyclin G-luciferase, mdm2 luciferase, p21 luciferase, and bax luciferase (16).

RESULTS

c-Abl Enhances the Transcriptional Activity of p53—c-Abl can enhance the ability of p53 to induce the mdm2 promoter and a synthetic responsive element (10, 11). Here we show that this cooperative effect holds true for other promoters. H1299 lung carcinoma cells lacking p53 were transfected with p53 alone or together with c-abl, and the luciferase reporter gene was driven by either the cyclin G or the mdm2 promoter (16). Activation of both promoters was increased by c-Abl more than 2-fold (Fig. 1A). Similar results were obtained using the p21 and bax promoters (data not shown). This enhancement of p53 transcriptional activity was independent of the kinase activity of c-Abl (data not shown), consistent with previous findings (10, 11).
c-Abl Neutralizes Mdm2 Effect on p53

We next tested whether this cooperation occurs also at physiological levels of p53 and c-Abl. This was tested in mouse embryonic fibroblast cell lines, derived from c-abl null mice, which express either lacZ (abl/abl + LacZ) or wild type c-abl (abl/+ + abl). The expression of c-abl protein in the c-abl reconstituted lines was found to be lower than that of endogenous c-Abl in NIH3T3 control cell line (Fig. 1 panel I, confirming that it is within the physiological range. When assayed with a p21+/+/# luciferase reporter gene, p53 activity was 2.5-fold higher in the c-abl reconstituted line than in the lacZ expressing cells (Fig. 1B). This effect was independent of c-abl kinase activity (data not shown). It should be noted that the luciferase activities of CMV-driven reporter control were similar in both lines (Fig. 1B, panels II). These results imply that c-Abl can positively modulate the transcriptional activity of p53 at physiological levels of both proteins.

C-Abl Stabilizes the p53 Protein—The mechanism by which p53 transcriptional activity is enhanced by c-Abl (Fig. 1 and Refs. 10 and 11) is yet to be elucidated. Because p53 is regulated largely at the protein level (reviewed in Refs. 9 and 17), we investigated whether c-Abl can affect p53 expression level. H1299 cells were transfected with a low amount of a p53 expression vector either alone or together with increasing amounts of c-abl expression vector, and the steady state expression levels of p53 were determined. The expression of p53 was elevated by c-abl in a dose-dependent manner (Fig. 2, lanes 1–3). This effect was observed at different inputs of p53 plasmids (data not shown). A similar elevation of p53 by c-abl was observed in Saos-2, an osteosarcoma cell line (data not shown), supporting the generality of this effect.

The kinase defective c-Abl, c-abl K290R, was also able to elevate p53 levels in a dose-dependent manner (Fig. 2, lanes 4–6), although to a lesser extent than that observed with wt c-Abl (e.g. compare lane 2 versus 5). Thus, c-Abl kinase activity is not essential for the elevation of p53 expression level, although it may have a contributory effect, suggesting that c-Abl may elevate p53 expression through more than one mechanism.

C-Abl Blocks Mdm2-mediated Degradation of p53—Our finding raised the intriguing possibility that c-Abl elevates p53 level by overcoming its destabilization by Mdm2. This assumption was tested in H1299 cells by determining the effect of c-Abl on the ability of Mdm2 to promote p53 degradation. Although the level of the p53 protein was markedly reduced by Mdm2 (Fig. 3A, panel I, lane 1 versus lane 2), in the presence of c-Abl the p53 protein was largely protected from degradation (Fig. 3A, panel I, lane 3). The same results were obtained in Saos-2 cells (data not shown). Like wt c-Abl, the kinase defective c-Abl K290R was able to neutralize the promotion of p53 degradation by Mdm2 (Fig. 3B). Thus, c-Abl protects p53 from destabilization by Mdm2 in a kinase-independent manner.

The involvement of Mdm2 in the stabilization of p53 by c-Abl was further examined by using two p53 mutants, p53Gln14, Ser19 and p53Gln22,Ser23, which do not bind Mdm2 (18) and are resistant to its destabilizing effect (4, 5). If c-Abl stabilizes p53 by neutralizing Mdm2, the expression level of these mutants should not be elevated by c-Abl. Indeed, unlike wt p53, p53Gln22,Ser23 was not stabilized by c-Abl (Fig. 3C). Similar results were obtained with p53Gln14,Ser19 (data not shown). These results further support the notion that c-Abl stabilizes p53 by preventing its degradation by Mdm2.

p53 Binds Mdm2 in the Presence of c-Abl—The finding that c-Abl neutralizes Mdm2-promoted p53 degradation raised the possibility that c-Abl may prevent the interaction between p53 and Mdm2, which is essential for p53 degradation (4, 5). This conjecture was tested by a co-immunoprecipitation assay. To increase the sensitivity of this assay a mutant form of human Mdm2 (Hdm2), Hdm2ΔRING, lacking the RING finger domain, was used. This mutant binds p53 without promoting its degradation (Fig. 4B and Ref. 5), thereby allowing the detection of stable p53–Hdm2 complexes. H1299 cells were transfected with p53 and Hdm2ΔRING in the presence or absence of c-Abl. Larger amounts of expression plasmids were used to obtain amounts of p53 and Hdm2 sufficient for detection. With such
amounts of DNA the p53 protein is stable (4), explaining why further stabilization by c-Abl is only marginal (Fig. 4B, lane 3 versus lane 4). In fact, the amount of co-immunoprecipitated Hdm2ΔRING was even larger in the presence of c-Abl (Fig. 4A, lane 4), correlating with the elevation in Hdm2ΔRING expression (Fig. 4D). Thus, c-Abl does not interfere with Mdm2-p53 complex formation.

**c-Abl Relieves Mdm2-mediated Inhibition of p53 Activity**—The significant effect of c-Abl on Mdm2-mediated degradation of p53 prompted us to evaluate its biological consequences. Since c-Abl prevents the degradation of p53 by Mdm2, it is conceivable that c-Abl may promote p53 activities even in the presence of Mdm2. This prediction was tested in two functional assays. First, we determined whether c-Abl can relieve the inhibitory effect of Mdm2 on the transcriptional activity of p53. H1299 cells were transfected with p53 alone (20 ng) or together with the indicated combinations of mdm2 (70 ng) and c-abl (4 µg), along with the cyclin G luciferase reporter plasmid (1 µg). Luciferase activity is shown in arbitrary units, along with the standard deviation of triplicates.

**FIG. 5.** c-Abl overcomes the inhibitory effect of Mdm2 on the transcriptional activity of p53. H1299 cells were transfected with p53 alone (20 ng) or together with the indicated combinations of mdm2 (70 ng) and c-abl (4 µg), along with the cyclin G luciferase reporter plasmid (1 µg). Luciferase activity is shown in arbitrary units, along with the standard deviation of triplicates.
of the inhibitory effect of Mdm2 by c-Abl renders p53 transcriptionally active.

The ability of c-Abl to overcome the inhibitory effect of Mdm2 on p53-mediated apoptosis (15, 19) was evaluated in Saos-2 cells, using a transient transfection assay. The induction of apoptosis by p53 was reduced more than 2-fold by co-expression of mdm2 (Fig. 6), which agrees with previous studies using HeLa (15). However, co-expression of c-abl abolished the effect of mdm2. Under these conditions c-Abl alone does not affect p53-mediated apoptosis to any significant extent (Fig. 6). Thus, c-Abl can alleviate the negative effect of Mdm2 on p53-mediated apoptosis.

**DISCUSSION**

In this study we have investigated the mechanisms underlying the cooperation between the growth inhibitory proteins p53 and c-Abl. The synergistic effect of c-Abl on p53 transcriptional activation (10, 11) has been confirmed and extended to include other physiological p53 responsive promoters. Importantly, this synergy was demonstrated between endogenous p53 and c-Abl at physiological protein levels, hence supporting its biological relevance. The enhancement of p53-mediated transactivation by c-Abl is largely due to the ability of c-Abl to increase the steady state level of p53 (Fig. 2). This is achieved by neutralizing Mdm2-mediated degradation of p53 (Fig. 3). This novel role for c-Abl provides a likely mechanistic explanation for the cooperation between these two proteins (Fig. 1 and Refs. 10 and 11). This explanation is supported by the findings that c-Abl neutralizes the inhibitory effect of Mdm2 on p53-mediated transactivation and apoptosis (Figs. 5 and 6). However, we cannot exclude the possibility that c-Abl may also enhance the biochemical activity of p53. The neutralization of Mdm2 is important for extending the time window during which p53 remains functional, especially under conditions where there is no apparent time lag between the induction of genes mediating growth suppression and the induction of mdm2 (20). As shown here, c-Abl neutralizes Mdm2, thereby providing the time delay required for p53 to exert its biological effects. In this manner, c-Abl can modulate the extent and duration of the p53 response.

The mechanisms by which c-Abl neutralizes the inhibitory effects of Mdm2 are unclear. Since c-Abl is mostly nuclear, it may prevent Mdm2-mediated cytoplasmic shuttling of p53, which is essential for p53 degradation (21). Alternatively, c-Abl may neutralize p53 destabilization by interfering with the degradation process. The oncogenic Ber-Abl can promote the ubiquitin-mediated degradation of Abi (22). As c-Abl and Ber-Abl have antagonistic effects on cell growth, they may also have opposite effects on protein degradation.

Neutralizing the inhibitory effect of Mdm2 may prove to be an efficient and common mechanism by which p53 is regulated. Indeed, recent reports showed that ARF-INK4a (p19Arf) cooperates with p53 by overcoming the inhibitory effects of Mdm2 (reviewed in Ref. 23). The p19Arf pathway is induced by several oncogenes, such as E1A (Ref. 23 and references therein). In addition, E1A can also stabilize and activate p53 by selectively blocking the induction of mdm2 gene expression by p53 (24) as well as by abrogating the interaction between Mdm2 and p300, which can facilitate p53 degradation (25). It is yet to be determined whether c-Abl interferes directly with Mdm2 function or whether it employ p19Arf, p300, or yet another protein to neutralize Mdm2. Nevertheless, this novel function of c-Abl is believed to be important for its growth suppression function.

**Acknowledgments**—We thank A. J. Levine, A. Zauberman, J. Reed, J. Wang, R. Maya, and B. Vogelstein for the generous gift of plasmids and D. Lane, S. Pikies, and J. Wang for the gift of antibodies. We thank Dr. Sue Moody-Haupt for critical comments.

**REFERENCES**

1. Gottlieb, T. M., and Oren, M. (1996) *Biochim. Biophys. Acta* 1287, 77–102
2. Levine, A. J. (1997) *Cell* 88, 323–331
3. Momand, J., and Zambetti, G. P. (1997) *J. Cell. Biochem.* 64, 343–352
4. Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997) *Nature* 387, 296–299
5. Kubbutat, M. H. G., Jones, S. N., and Vousden, K. H. (1997) *Nature* 387, 299–303
6. Honda, R., and Yasuda, H. (1999) *EMBO J.* 18, 22–27
7. 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
8. Kharbanda, S., Pandey, P., Jin, S., Inoue, S., Bharti, A., Yuan, Z. M., Weichselbaum, R., Weaver, D., and Kufe, D. (1997) *Nature* 386, 732–735
9. Prives, C. (1996) *Cell* 85, 6–8
10. Goga, A., Liu, X., Hambuch, T. M., Senechal, K., Major, E., Berk, A. J., Witte, O. N., and Sawyer, C. L. (1995) *Oncogene* 11, 791–799
11. Yuan, Z.-M., Huang, Y., Whang, Y., Sawyers, C., Weichselbaum, R., Kharbanda, S., and Kufe, D. (1996) *Nature* 382, 272–274
12. Yuan, Z.-M., Huang, Y., Fan, M. M., Sawyers, C., Kharbanda, S., and Kufe, D. (1996) *J. Biol. Chem.* 271, 26457–26460
13. Wen, S. T., Jackson, P. K., and Van Etten, R. A. (1996) *EMBO J.* 15, 1583–1595
14. Yuan, Z.-M., Huang, Y., Ishiko, T., Kharbanda, S., Weichselbaum, R., and Kufe, D. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 1437–1440
15. Haupt, Y., Barak, Y., and Oren, M. (1996) *EMBO J.* 15, 1596–1606
16. Friedlander, P., Haupt, Y., Prives, C., and Oren, M. (1996) *Mol. Cell. Biol.* 16, 4961–4971
17. Giaccia, A. J., and Kastan, M. B. (1998) *Genes Dev.* 12, 2973–2983
18. Lin, J., Chen, J., Elenbaas, B., and Levine, A. J. (1994) *Genes Dev.* 8, 1235–1246
19. Chen, J., Wu, X., Lin, J., and Levine, A. J. (1996) *Mol. Cell. Biol.* 16, 2445–2452
20. Reineke, V., and Lozano, G. (1998) *Oncogene* 15, 1527–1534
21. Roth, J., Dobelestein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998) *EMBO J.* 17, 554–564
22. Dai, Z., Quackenbush, R. C., Courtney, K. D., Grove, M., Cortez, D., Reuther, G. W., and Pendergast, A. M. (1996) *Genes Dev.* 10, 1415–1424
23. Sherr, C. J. (1998) *Genes Dev.* 12, 2984–2991
24. Thomas, A., and White, E. (1998) *Genes Dev.* 12, 1975–1985
25. Grossman, S. R., Perez, M., Kung, A. L., Joseph, M., Mansur, C., Xiao, Z. X., Kumar, S., Howley, P. M., and Livingston, D. M. (1998) *Mol. Cell* 2, 405–415