Regulation of DNA-dependent Protein Kinase by the Lyn Tyrosine Kinase*

(Received for publication, April 28, 1998, and in revised form, July 28, 1998)

Shailendra Kumar‡, Pramod Pandey‡, Ajit Bharti‡, Shengfang Jin§, Ralph Weichselbaum§, David Weaver§, Donald Kufe‡, and Surender Kharbanda‡

From the ‡Department of Adult Oncology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115, the ¶Department of Radiation and Cellular Biology, University of Chicago, Chicago, Illinois 60637, and the §Department of Microbiology and Molecular Genetics, Harvard Medical School, Boston, Massachusetts 02115

The Src-like protein-tyrosine kinase Lyn is activated by ionizing radiation and certain other DNA-damaging agents, whereas the DNA-dependent protein kinase (DNA-PK), consisting of the catalytic subunits (DNA-PKcs) and Ku DNA-binding components, requires DNA double-stranded breaks for activation. Here we demonstrate that Lyn associates constitutively with DNA-PKcs. The SH3 domain of Lyn interacts directly with DNA-PKcs near a leucine zipper homology domain. We also show that Lyn phosphorylates DNA-PKcs but not Ku in vitro. The interaction between Lyn and DNA-PKcs inhibits DNA-PKcs activity and the ability of DNA-PKcs to form a complex with Ku/DNA. These results support the hypothesis that there are functional interactions between Lyn and DNA-PKcs in the response to DNA damage.

Mammalian cells respond to DNA damage with cell cycle arrest, activation of DNA repair, and, in the event of irreparable lesions, the induction of apoptosis. The signals controlling responses to genotoxic stress, while of importance to mutagenesis and treatment with certain anti-cancer agents, remain unclear. Certain insights, however, have been derived from the finding that DNA-damaging agents activate the c-Abl protein-tyrosine kinase (PTK) (1–4). c-Abl is detectable in a nuclear complex with the DNA-dependent protein kinase (DNA-PK) and is activated in part in the response to ionizing radiation (IR) by a DNA-PK-dependent mechanism (5). Activation of c-Abl is associated with binding to the p53 tumor suppressor and the induction of growth arrest in G1 phase through down-regulation of the cyclin-dependent kinase 2 (Cdk2) (6, 7). Other studies have demonstrated that c-Abl contributes to DNA damage-induced apoptosis (8, 9). These findings have supported a role for c-Abl in regulating the growth arrest and apoptotic responses to genotoxic stress.

c-Abl is present in a nuclear complex that includes the Src-like Lyn PTK (10). Lyn, like c-Abl, is activated by IR and other DNA damaging agents (11–14). The activation of nuclear Lyn by DNA damage is associated with binding of Lyn to Cdc2 (11–14). Lyn phosphorylates Cdc2 on Tyr-15 and thereby inhibits Cdc2 activity (11–14). Whereas activation of Cdc2 in a complex with cyclin B is required for the transition of cells from G2 to M phase (15), inhibition of Cdc2 by Lyn could contribute in part to the arrest at G2/M phase following exposure to DNA damaging agents. Alternatively, binding of Lyn to Cdc2 may prevent the interaction of Cdc2 with proteins such as c-Src that play a functional role in mitosis (16). Other studies have indicated that the activation of Lyn is not restricted to cells in G2. In this context, arrest of cells in G2/M phase by 1-b-d-arabinofuranosylcytosine is associated with activation of Lyn and binding of Lyn to Cdk2 (17). Thus, the available evidence suggests that the Lyn PTK, like c-Abl, plays a role in the cell cycle arrest response to DNA damaging agents.

DNA-PK, a complex of three proteins, is involved in the repair of DNA double-stranded breaks, V(D)J recombination, and transcription (18–22). The 470-kDa catalytic subunit of DNA-PK (DNA-PKcs) is activated by binding with the 70- and 80-kDa Ku heterodimer to sites of DNA damage (23–26). Under some conditions, DNA-PK is a self-contained kinase that is activated by direct interaction with double-stranded DNA, whereas Ku stabilizes binding of DNA-PK to DNA ends (21, 27). Among the many substrates of activated DNA-PKcs are p53, c-Abl, and Cdc2 (5, 19, 26). Autophosphorylation of DNA-PKcs inhibits its activity by inducing the dissociation of DNA-PKcs from DNA (28). Phosphorylation of DNA-PKcs by c-Abl also inhibits the ability of DNA-PK to form a complex with DNA (5). c-Abl, like Ku, associates with DNA-PKcs near the kinase homology region (29). c-Abl phosphorylates DNA-PKcs in the C-terminal region and thereby dissociates DNA-PKcs from the Ku-DNA complex (29). Otherwise, little is known about the regulation of DNA-PK activity.

The findings that c-Abl forms a nuclear complex with Lyn (10) and that c-Abl interacts with DNA-PK prompted studies on a potential interaction between Lyn and DNA-PK. The present results demonstrate that Lyn binds directly to DNA-PK. We also show that Lyn phosphorylates DNA-PKcs and inhibits DNA-PKcs activity.

MATERIALS AND METHODS

Cell Culture—U-937 monoblastic leukemia cells (ATCC, Rockville, MD) were grown in RPMI 1640 media containing 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, 100 mg/ml streptomycin, and 2 mM l-glutamine. Irradiation was performed at room temperature using a Gammacell-1000 (Atomic Energy of Canada, Ottawa, ON, Canada) under aerobic conditions with 150 Gy source emitting at a fixed dose rate of 0.76 Gray/min.

Immunoprecipitation and Immunoblot Analysis—Preparation of cell lysates and immunoprecipitations were performed as described (3). Soluble proteins were incubated with anti-Lyn (Upstate Biotechnology Inc., Lake Placid, NY) or anti-DNA-PK (Upstate Biotechnology Inc.) for 1 h and precipitated with protein A-Sepharose for an additional 1 h. The
FIG. 1. Association of DNA-PKcs with Lyn. A, lysates from U-937 cells were subjected to immunoprecipitation with PIRS or anti-Lyn. Immunoprecipitates were analyzed by immunoblotting with anti-DNA-PK. Cell lysate was also subjected to immunoprecipitations with anti-DNA-PK as a positive control. B, U-937 cells were treated with 20-gray IR and harvested at the indicated times. Lysates were then subjected to immunoprecipitation with anti-Lyn. Lysate was also used directly as a positive control. Immunoprecipitates were analyzed by immunoblotting with anti-DNA-PK. C, schematic diagrams of various Lyn constructions. D, U-937 cell lysate was incubated with GST-Lyn 131–243, GST-Lyn 1–243, GST-Lyn 1–131, or GST-Lyn 27–131 fusion proteins. The adsorbates were analyzed by immunoblotting with anti-DNA-PK. IB, immunoblot.

RESULTS AND DISCUSSION

To determine whether nuclear Lyn associates with DNA-PK, we subjected anti-Lyn immunoprecipitates to immunoblot analysis with anti-DNA-PK antibodies. Whereas immunoprecipitates obtained with PIRS had no detectable DNA-PKcs immunoprecipitation with the anti-Lyn antibody revealed the presence of complexes containing DNA-PKcs (Fig. 1A). To evaluate the stoichiometry of interaction between DNA-PKcs and Lyn, we subjected U-937 cell lysates to immunoprecipitation with anti-Lyn and analyzed the precipitates by immunoblotting with anti-DNA-PK. Signal intensities from before and after anti-Lyn immunoprecipitation were compared by laser densitometric scanning. The results demonstrate that approximately 25% of DNA-PKcs is associated with Lyn (data not shown). Because exposure of cells to IR is associated with activation of both Lyn (11) and DNA-PK (24), we also investigated whether IR affects the interaction between these proteins. IR treatment was associated with a reproducible increase in the association of Lyn with DNA-PKcs, to some extent (Fig. 25655).
1B). To confirm binding of Lyn and DNA-PKcs, cell lysates were incubated with a GST fusion protein prepared from the 1–243 amino acid fragment of Lyn that includes a unique N-terminal region, Src homology 3 (SH3) and SH2 domains but not kinase domains (30) (Fig. 1C). Adsorbates obtained with GST-Lyn 1–243 but not with GST demonstrated binding of DNA-PKcs (Fig. 1D and data not shown). Adsorbates obtained with GST-Lyn 1–131 and GST-Lyn 27–131 but not with GST-Lyn 131–243 also revealed specific binding of DNA-PKcs to the Lyn SH3 domain (Fig. 1D). These findings indicate that the SH3 domain of Lyn contributes to the association with DNA-PK.

To assess whether Lyn binds directly to DNA-PKcs, we incubated purified DNA-PKcs with Lyn. Anti-Lyn immunoprecipitates were analyzed by immunoblotting with anti-DNA-PK. In contrast to PIRS, immunoblotting with anti-Lyn revealed specific DNA-PKcs polypeptides. In the presence of DNA, DNA-PKcs/Ku phosphorylated Lyn 1–131 and GST-Lyn but not with GST demonstrated binding of DNA-PKcs to the Lyn SH3 domain (Fig. 1D). These findings indicate that the direct interaction of Lyn and DNA-PKcs occurs by direct interaction of the Lyn SH3 domain with DNA-PKcs amino acids 1744–1755; fragments 11 and 14) in DNA-PKcs, the region, Src homology 3 (SH3) and SH2 domains but not kinase domains (30). Lyn phosphorylation of DNA-PKcs did not require DNA-bound DNA-PK. Also, as a positive control, DNA-PK was incubated with DNA beads to show autophosphorylation of DNA-PKcs (Fig. 3A). To confirm Lyn-dependent phosphorylation of DNA-PKcs, we incubated purified DNA-PKcs with active or HI Lyn in kinase buffer containing cold ATP. The phosphorylated products were analyzed by immunoblotting with anti-P-Tyr. The results demonstrate Lyn-dependent tyrosine phosphorylation of DNA-PKcs (Fig. 3B). DNA-PKcs forms a complex with the 70- and 80-kDa Ku heterodimer (26, 32), and thus we also incubated Lyn with purified Ku in a kinase reaction. In contrast to DNA-PKcs, there was no detectable phosphorylation of Ku (Fig. 3C). These findings indicate that Lyn phosphorylates DNA-PKcs and not Ku.

The activation of DNA-PK by binding of DNA-PKcs to Ku/DNA is associated with phosphorylation of p53 (19). Therefore to assess the effect of Lyn on DNA-PK activity, we incubated purified DNA-PKcs/Ku/DNA complexes with active or inactive Lyn and assessed DNA-PKcs activity using GST-p53 as a substrate. In the presence of DNA, DNA-PKcs/Ku phosphorylated GST-p53 (Fig. 3D, lane 1). By contrast, addition of either active or inactive Lyn to the reaction inhibited GST-p53 phosphorylation (Fig. 3D, lanes 2 and 3). In the absence of DNA-PK, Lyn had little effect on phosphorylation of GST-p53 (Fig. 3D, lane 4). These findings indicate that the direct interaction of Lyn with DNA-PKcs, and not necessarily the Lyn kinase function, contributes to the inactivation of DNA-PKcs. To further assess the functional significance of the activation of Lyn and DNA-PK, the DNA-PKcs/Ku complex was bound to DNA beads and incubated with active or HI Lyn. The reactions included wortmannin to inhibit DNA-PKcs autophosphorylation and thereby inhibit autodissociation from DNA (28). Incubation with kinase-active or kinase-inactive Lyn resulted in release of DNA-PKcs from the beads into the supernatant (Fig. 4B). By contrast, addition of the MEK1 kinase (Fig. 4C). Similar results were obtained with the MEK1 kinase (Fig. 4C). DNA-PKcs released from Ku/DNA in the presence of Lyn as compared with release of the DNA-PKcs/Ku complex from DNA was assessed by immunoautoradiography of proteins in the supernatant and bound to the beads. The results demonstrate that approximately 40% of DNA-PKcs is released from Ku in the presence of Lyn (Fig. 4D). By contrast, only 2–4% of DNA-PKcs/Ku complexes were released from the DNA beads in the presence of Lyn (Fig. 4D). These findings demonstrate that interaction of Lyn and DNA-PKcs results in dissociation of DNA-PKcs and Ku, and thereby.
directs inhibition of DNA-PKcs activity.

DNA-PK is essential in the repair of DNA double-stranded breaks that form in irradiated cells (20, 33, 34). Autophosphorylation inactivates DNA-PK by a mechanism in which DNA-PKcs dissociates from Ku (28). Other studies have shown that c-Abl negatively regulates DNA-PK in the response to DNA damage (5). The present studies demonstrate that DNA-PK is also regulated by Lyn. DNA-PK constitutively associates with Lyn by direct binding of the Lyn SH3 domain to an internal region of DNA-PKcs that includes a leucine zipper. Lyn also phosphorylates DNA-PKcs. The in vitro findings indicate that the direct binding of Lyn to DNA-PKcs is sufficient to inhibit DNA-PKcs activity. Thus, constitutive binding of Lyn and DNA-PKcs could regulate the accessibility of certain pools of DNA-PKcs for interaction with Ku/DNA complexes. Lyn-mediated phosphorylation of DNA-PKcs represent another level of DNA-PKcs regulation. These results are in concert with the demonstration that the interaction between DNA-PKcs and Lyn induces the dissociation of DNA-PKcs from the Ku/DNA complex and thereby inhibits DNA-PKcs activity. The activation of Lyn by IR and other DNA damaging agents contributes to the down-regulation of Cdc2 (13–16), indicating that Lyn is an effector of cell cycle progression in the response to DNA damage. The present findings support a function for Lyn in the regulation of DNA repair. Accordingly, interactions between Lyn and DNA-PKcs may play a role in releasing DNA-PKcs from Ku/DNA complexes after repair to permit relocation at new sites of DNA damage.

Acknowledgments—We thank Dr. Stephen Jackson for providing human DNA-PK fragments, Dr. S. P. Lees-Miller for providing purified Ku, Dr. Vimla Band for the GST-p53 cDNA construct, Dr. Hamid Band for anti-Ku antibody (GE 9.2), and Dr. J. Cambier for GST-Lyn constructs. We also thank Andrew Place, Atsuko Nakazawa, and Rebecca Farber for excellent technical assistance.

REFERENCES
1. Kharbanda, S., Ren, R., Pandey, P., Shafman, T. D., Feller, S. M., Weichselbaum, R. R., and Kufe, D. W. (1995) Cancer Res. 55, 785–788
2. Kharbanda, S., Pandey, P., Ren, R., Feller, S., Mayer, B., Zon, L., and Kufe, D.
Regulation of DNA-PK by the Lyn Tyrosine Kinase

3. Kharbanda, S., Bharti, A., Pei, D., Wang, J., Pandey, P., Ren, R., Weichselbaum, R., Walsh, C. T., and Kufe, D. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6898–6901

4. Liu, Z.-G., Baskaran, R., Lea-Chou, E. T., Wood, L., Chen, Y., Karin, M., and Wang, J. Y. J. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 6898–6901

5. Yuan, Z.-M., Huang, Y., Kharbanda, S., Weaver, D., and Kufe, D. (1999) Biochim. Biophys. Acta 1333, 1–7

6. Yuan, Z.-M., Kharbanda, S., Saleem, A., Shafman, T., Emoto, Y., Weichselbaum, R., Woodgett, J., Avruch, J., Kyriakis, J., and Kufe, D. (1995) Mol. Cell. Biol. 15, 5700–5706

7. Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. S. I., Barron, G. M., and Allalunis-Turner, J. (1995) Science 267, 1183–1185

8. Gottlieb, T. M., and Jackson, S. P. (1993) Cell 72, 131–142

9. Gottlieb, T. M., and Jackson, S. P. (1993) Cell 72, 131–142

10. Carter, T., Vancuero, I., Sun, I., Lou, W., and DeLeon, S. (1990) Mol. Cell. Biol. 10, 6460–6471

11. Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. S. I., Barron, G. M., and Allalunis-Turner, J. (1995) Science 267, 1183–1185

12. Lees-Miller, S. P., Chen, Y., and Anderson, C. W. (1990) Mol. Cell. Biol. 10, 6472–6481

13. Jackson, S. P., MacDonald, J. J., Lees-Miller, S., and Tjian, R. (1990) Cell 63, 155–165

14. Boubnov, N. V., and Weaver, D. T. (1995) Mol. Cell. Biol. 15, 5700–5706

15. Nurse, P. (1990) Nature 344, 503–507

16. Shenoy, S., Chackalaparampil, I., Bagrodia, S., Lin, P.-H., and Shalloway, D. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 7237–7241

17. Yuan, Z.-M., Huang, Y., Kharbanda, S., and Kufe, D. (1999) Biochim. Biophys. Acta 1333, 1–7

18. Carter, T., Vancuero, I., Sun, I., Lou, W., and DeLeon, S. (1990) Mol. Cell. Biol. 10, 6460–6471

19. Lees-Miller, S. P., Sakaguchi, K., Ullrich, S. J., Appella, E., and Anderson, C. W. (1992) Mol. Cell. Biol. 12, 5041–5049

20. Hartley, K. O., Gell, D., Smith, G. C. M., Zhang, H., Divecha, N., Connely, M. A., Admon, A., Lees-Miller, S. P., Anderson, C. W., and Jackson, S. P. (1995) Cell 82, 849–856

21. Chu, G. (1999) J. Biol. Chem. 274, 24097–24100

22. Jackson, S. P., MacDonald, J. J., Lees-Miller, S., and Tjian, R. (1990) Cell 63, 155–165

23. Nurse, P. (1990) Nature 344, 503–507

24. Lees-Miller, S. P., Godbout, R., Chan, D. W., Weinfeld, M., Day, R. S. I., Barron, G. M., and Allalunis-Turner, J. (1995) Science 267, 1183–1185

25. Gottlieb, T. M., and Jackson, S. P. (1993) Cell 72, 131–142

26. Lees-Miller, S. P., Chen, Y., and Anderson, C. W. (1990) Mol. Cell. Biol. 10, 6472–6481

27. Hammarsten, O., and Chu, G. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 525–530

28. Chan, D. W., and Lees-Miller, S. P. (1996) J. Biol. Chem. 271, 8936–8941

29. Jin, S., Kharbanda, S., Mayer, B., Kufe, D., and Weaver, D. T. (1997) J. Biol. Chem. 272, 24763–24766

30. Pleiman, C. M., Clark, M. R., Timson Gauen, L. K., Winitz, S., Coggeshall, K. M., Johnson, G. L., Shaw, A. S., and Cambier, J. C. (1993) Mol. Cell. Biol. 13, 5877–5887

31. Kharbanda, S., Saleem, A., Shafman, T., Emoto, Y., Weichselbaum, R., Woodgett, J., Avruch, J., Kyriakis, J., and Kufe, D. (1995) J. Biol. Chem. 270, 18571–18574

32. Anderson, C. W., and Lees-Miller, S. P. (1992) Crit. Rev. Eukaryotic Gene Expression 2, 283–314

33. Jackson, S. P., and Jeggo, P. A. (1995) Trends Biochem. Sci. 20, 412–415

34. Weaver, D. T. (1995) Trends Genet. 11, 388–392