Characterization of Two DNA Double-stranded Break Repair-deficient Cell Lines That Express Inactive DNA-dependent Protein Kinase Catalytic Subunits*

(Received for publication, October 24, 1996, and in revised form, January 30, 1997)

Scott R. Peterson‡§, Murray Stackhouse‡, Mary Jo Waltman‡, Fanqing Chen‡, and David J. Chen‡

From the Life Sciences Division, Los Alamos National Laboratory, Los Alamos, New Mexico 87545, the Division of Radiation Biology and Gene Therapy Program, University of Alabama, Birmingham, Alabama 35233, and the Division of Radiobiology and Biodosimetry, National Institute of Radiological Sciences, Chiba 263, Japan

The DNA-dependent protein kinase (DNA-PK) is a trimeric enzyme consisting of a 460-kDa catalytic subunit (DNA-PKcs) and a heterodimeric regulatory complex called Ku, which is comprised of 70 (Ku70) and 86 (Ku80) kDa subunits. Mutations that affect the expression of the catalytic or Ku80 subunits of DNA-PK disrupt both V(D)J recombination and DNA double-stranded break repair pathways. In this report, we show that two previously uncharacterized rodent cell lines that are defective in DNA double-stranded break repair express catalytically inactive DNA-PK. The DNA-PKcs from the DNA double-stranded break repair mutant cell lines IRS-20 and SX-9 assembles on double-stranded DNA but fails to function as a protein kinase. In addition to the kinase defect, the abundance of the DNA-PKcs from both of these cell lines is reduced relative to wild-type controls. These results suggest that the DNA-PKcs gene from each of these cell lines contains mutations that inactivate the enzymatic activity and the expression or stability of the gene product. These data further strengthen the hypothesis that DNA-PK-mediated protein phosphorylation is a necessary component of the DNA double-stranded break repair pathway.

The rejoining of double-stranded DNA breaks induced by ionizing radiation or occurring as intermediates of V(D)J recombination is performed via a biochemical pathway that includes the DNA-dependent protein kinase holoenzyme. DNA-PK is a trimeric complex consisting of a DNA-binding component made up of the 70 and 86 kD subunits of the Ku autoantigen (1, 2) and a catalytic subunit of approximately 460 kDa (3). Cells from the x-ray-sensitive complementation group IRS-20 (20, 21) and SX-9 (22) both express DNA-PK catalytic subunits that can assemble on double-stranded DNA but lack detectable protein kinase activity. The expression and activity of the Ku subunits of DNA-PK are normal in each cell line, and DNA-PK catalytic and enzymatic activity and the expression or stability of the Ku and DNA-PKcs subunits of DNA-PK (18, 19). Based on these data, it has been proposed that DNA-PK binds to double-stranded DNA breaks produced in the cell by DNA-damaging agents or during V(D)J recombination. The DNA-bound holoenzyme could then participate in the DNA-rejoining process by phosphorylating Ku and other protein factors that are colocalized with the kinase at the site of the strand breaks.

In this report, we present data that further demonstrates the importance of DNA-PK in the DNA double-stranded break repair process by showing that two DNA double-stranded break repair-deficient cell lines have defects that disrupt the catalytic activity of DNA-PK. The CHO cell lines IRS-20 (20, 21) and SX-9 (22) both express DNA-PK catalytic subunits that can assemble on double-stranded DNA but lack detectable protein kinase activity. The expression and activity of the Ku subunits of DNA-PK are normal in each cell line, and DNA-PK activity can be restored to both the IRS-20 and SX-9 cell extracts by addition of purified DNA-PKcs. Transfer of human chromosome 8, which contains the DNA-PKcs gene, can also rescue the kinase defect of the IRS-20 cells. These data are consistent with the IRS-20 and SX-9 cells being in the same complementation group as the mouse scid and CHO V3 cells and support the hypothesis that DNA-PK-mediated protein phosphorylation is an essential component of the DNA double-stranded break repair pathway.

EXPERIMENTAL PROCEDURES

Cell Growth Conditions and Cell Extract Preparation—Cells were grown as monolayer cultures (10B2, IRS-20, IRS-20 (Neo8), M10, LX380, SL3147, and H5) (22–24) in a humidified, 5% CO₂ atmosphere...
with α-minimal essential medium supplemented with 10% heat-inactivated calf serum or in suspension (SR1, SX9, S10) (23) with RPMI 1640 medium supplemented with 10% heat-inactivated calf serum. Cell extracts were prepared as described previously (6). Briefly, cells were swelled in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.9, 5 mM KCl, 1.5 mM MgCl₂, 0.5 mM dithiothreitol, 20 µg/ml phenylmethylsulfonil fluoride, 1 mM peptin, 1 mM leupeptin, 1 mM aprotinin) for 30 min at 4 °C and then lysed using a Dounce homogenizer with a loose fitting pestle. Nuclei were pelleted by centrifugation for 5 min. at 2000 × g and extracted for 30 min. on ice using nuclear extraction buffer (50 mM Tris-HCl, pH 7.9, 0.5 mM NaCl, 20% glycerol, 10% sucrose, 2 mM EDTA, 1 mM dithiothreitol, 20 µg/ml phenylmethylsulfonil fluoride, 1 mM peptin, 1 mM leupeptin, 1 mM aprotinin) for 30 min at 25 °C and then washed with TM buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 20% glycerol) containing 100 mM KCl, 1 mM dithiothreitol, 20 µg/ml phenylmethylsulfonil fluoride, 10 µg/ml SBTI, 1 µg/ml each of leupeptin, pepstatin A, and aprotinin. The cytoplasmic and nuclear fractions were combined, and insoluble material was removed by centrifugation at 18,000 × g for 30 min. The extracts were then dialyzed against TM buffer (50 mM Tris-HCl, pH 7.9, 12.5 mM MgCl₂, 1 mM EDTA, 20% glycerol) containing 100 mM KCl, 1 mM dithiothreitol, 20 µg/ml phenylmethylsulfonil fluoride, 10 µg/ml SBTI, 1 µg/ml each of leupeptin, pepstatin A, and aprotinin. This allowed for the removal of weakly bound protein kinases by washing the DNA beads prior to initiating the kinase reactions.

We first tested the phosphorylation of RPA by DNA-PK using these assay conditions with purified HeLa cell DNA-PKcs (Fig. 1). As expected, in the absence of the Ku70/80 complex, phosphorylation of the 32-kDa RPA subunit by the DNA-PKcs was much lower than that observed for the holoenzyme (Fig. 1B, compare lanes b and c). This was likely due to the removal of the majority of the DNA-PKcs from the DNA beads during the washing step. To test whether the RPA kinase assay could discriminate DNA-PK activity from other kinases capable of phosphorylating RPA in cell extracts, we compared the RPA kinase activity in extracts prepared from wild-type and known DNA-PK mutant cell lines. RPA kinase activity was detected in cell extracts derived from the wild-type CHO cell line AA8, but was absent in the CHO K1-derived DNA-PKcs mutant cell line V3 (4) (Fig. 1C, compare lanes b and d). RPA kinase activity was restored to the V3 cell extract by the addition of 50 ng of purified DNA-PK catalytic subunit (Fig. 1C, lane f). Similarly, extracts prepared from the large T antigen immortalized scid mouse cell line SCVA2 (6) also lacked RPA kinase activity in this assay, whereas SCVA2 cells containing human chromosome 8 (SC (8)10) (29) had strong RPA kinase activity (Fig. 1D, compare lanes b and d). RPA kinase activity was restored to the SCVA2 cell extracts by addition of purified DNA-PKcs (Fig. 1D, lane f). To determine whether the RPA kinase assay was also sensitive to mutations that disrupt the activity of the Ku complex, we measured RPA phosphorylation using extracts derived from the Ku80 mutant cell line XR6S6C and XR6S6C cells that were engineered to express the human Ku80 protein (30). We found that RPA kinase activity was absent in the XR6S6C cell extracts but was present in extracts derived from the Ku80 expressing XR6S6C cells (Fig. 1E, compare lanes b and d). Since the RPA kinase assay was capable of discriminating between wild-type and known DNA-PK mutant cells, we used it to screen seven DNA double-stranded break repair-deficient rodent cell lines for DNA-PK kinase activity: IRS-20 (23), M10, LX380, SX9, and SX10 (22, 23), SL3147, and H5 (24). Of these cells, we found the CHO mutant IRS-20 and the mouse mammary carcinoma cell line SX9 lacked detectable RPA-kine activity. We chose to first characterize the IRS-20 cell line since it has recently been shown that DNA double-stranded break repair in these cells can be complemented by transfection of human chromosome 8. The human DNA-PKcs gene is located on chromosome 8 (31), suggesting that reduced expression or activity of the DNA-PKcs was responsible for the altered DNA-PK activity in the IRS-20 cell extracts. To determine whether this was true, we compared the DNA-bound RPA kinase activities of extracts prepared from the parental cell line

**RESULTS**

To screen previously uncharacterized DNA double-stranded break repair-deficient cell lines for mutations that affect DNA-PK, we developed an assay to measure the kinase activity of DNA-PK in cell extracts. In this assay, DNA-PK activity is determined by evaluating the phosphorylation of the 32-kDa subunit of recombinant human replication protein A (RPA) (26), which has previously been shown to be a substrate for DNA-PK in vitro (27, 28) and may also be phosphorylated by this enzyme in vivo in response to DNA damage (19). To enrich for DNA-PK from cell extracts, protein kinase reactions are first assembled on a DNA substrate covalently attached to agarose beads. This allows for the removal of weakly bound protein kinases by washing the DNA beads prior to initiating the kinase reactions.

We first tested the phosphorylation of RPA by DNA-PK using these assay conditions with purified HeLa cell DNA-PKcs (Fig. 1). As expected, in the absence of the Ku70/80 complex, phosphorylation of the 32-kDa RPA subunit by the DNA-PKcs was much lower than that observed for the holoenzyme (Fig. 1B, compare lanes b and c). This was likely due to the removal of the majority of the DNA-PKcs from the DNA beads during the washing step. To test whether the RPA kinase assay could discriminate DNA-PK activity from other kinases capable of phosphorylating RPA in cell extracts, we compared the RPA kinase activity in extracts prepared from wild-type and known DNA-PK mutant cell lines. RPA kinase activity was detected in cell extracts derived from the wild-type CHO cell line AA8, but was absent in the CHO K1-derived DNA-PKcs mutant cell line V3 (4) (Fig. 1C, compare lanes b and d). RPA kinase activity was restored to the V3 cell extract by the addition of 50 ng of purified DNA-PK catalytic subunit (Fig. 1C, lane f). Similarly, extracts prepared from the large T antigen immortalized scid mouse cell line SCVA2 (6) also lacked RPA kinase activity in this assay, whereas SCVA2 cells containing human chromosome 8 (SC (8)10) (29) had strong RPA kinase activity (Fig. 1D, compare lanes b and d). RPA kinase activity was restored to the SCVA2 cell extracts by addition of purified DNA-PKcs (Fig. 1D, lane f). To determine whether the RPA kinase assay was also sensitive to mutations that disrupt the activity of the Ku complex, we measured RPA phosphorylation using extracts derived from the Ku80 mutant cell line XR6S6C and XR6S6C cells that were engineered to express the human Ku80 protein (30). We found that RPA kinase activity was absent in the XR6S6C cell extracts but was present in extracts derived from the Ku80 expressing XR6S6C cells (Fig. 1E, compare lanes b and d). Since the RPA kinase assay was capable of discriminating between wild-type and known DNA-PK mutant cells, we used it to screen seven DNA double-stranded break repair-deficient rodent cell lines for DNA-PK kinase activity: IRS-20 (23), M10, LX380, SX9, and SX10 (22, 23), SL3147, and H5 (24). Of these cells, we found the CHO mutant IRS-20 and the mouse mammary carcinoma cell line SX9 lacked detectable RPA-kine activity. We chose to first characterize the IRS-20 cell line since it has recently been shown that DNA double-stranded break repair in these cells can be complemented by transfection of human chromosome 8. The human DNA-PKcs gene is located on chromosome 8 (31), suggesting that reduced expression or activity of the DNA-PKcs was responsible for the altered DNA-PK activity in the IRS-20 cell extracts. To determine whether this was true, we compared the DNA-bound RPA kinase activities of extracts prepared from the parental cell line

**REFERENCES**

1. J. Y.-D. Lin, M. C. Muhlmann-Diaz, M. A. Stackhouse, J. F. Robinson, G. E. Taccioli, and J. S. Bedford, manuscript submitted for publication.
10B2 and the mutant IRS-20 and IRS-20 cells containing human chromosome 8 (IRS-20(Neo8)). We found that the 32-kDa RPA subunit was phosphorylated in the 10B2 but not the IRS-20 kinase reactions (Fig. 2A, compare lanes b and d). RPA kinase activity was also detected in the IRS-20 (Neo8) kinase reactions (Fig. 2A, lane f) and in reactions performed using IRS-20 cell extracts that had been supplemented with purified DNA-PKcs (Fig. 2A, lane h).

We have shown previously that the CHO cell line V3 displays a severe reduction in the expression of the DNA-PKcs (6). To test whether this was also true for the IRS-20 cells, we measured the levels of both the DNA-PKcs and the Ku70 proteins in the IRS-20 cell extracts by immunoblot analysis using monoclonal antibodies specific for these proteins. Using this assay, we found the amount of DNA-PKcs in the IRS-20 extracts was reduced relative to cell extracts prepared from the parental cell line 10B2 (Fig. 2B, compare lanes a and b). In contrast, we found there was no difference in the abundance of the Ku70 protein when comparing these same cell extracts (Fig. 2B, compare lanes a and b), which is indicative of the status of both the Ku70 and Ku80 proteins (12, 30).

The results of the immunoblot analysis suggested to us that the reduced amount of DNA-PKcs subunit found in the IRS-20 mutant cell extracts could be responsible for the radiosensitive phenotype of these cells. This would be consistent with the defects observed in DNA double-stranded break repair-deficient rodent cells (5, 6). However, the level of DNA-PKcs expressed in the IRS-20 cells was much higher than that observed for the CHO V3 cells (Fig. 3A, compare lanes b and d). This indicated to us that in addition to the reduced level of DNA-PKcs expression, the IRS-20 cells might also have defects that affect the assembly of the DNA-PK holoenzyme on DNA or reduce the kinase activity of the enzyme. To test this, we measured the capacity of the DNA-PKcs from the parental 10B2 and the mutant IRS-20 cells to bind to DNA by measuring its retention on linearized DNA covalently linked to agarose beads. Using this assay we found that the amount of DNA-PKcs bound to the DNA-beads was proportional to its abundance in the cell extracts (Fig. 3A, lanes a and h), mutant IRS-20 (lanes c, d, g and h), and the IRS-20 cells transfected with human chromosome 8 (IRS-20(Neo8), lanes e and f). RPA was included in samples as indicated, and 50 ng of purified DNA-PKcs was included in samples loaded in lanes g and h. B, DNA-PKcs protein (lanes a and b) and Ku70 protein (lanes c and d) levels were determined by immunoblot analysis of extracts prepared from 10B2 and IRS-20 cells, as indicated. Protein samples were resolved by 6% SDS-PAGE and analyzed as described under “Experimental Procedures.” Arrows indicate the position of the DNA-PKcs and Ku70 subunits. The lower molecular weight band observed in the DNA-PKcs blot results from the cross-reactivity of the antibody with an unknown 200-kDa protein.
activity in a series of reactions prepared using 10B2 cell extract that was diluted with reaction buffer to give DNA-PK levels equivalent to those found in the IRS-20 reactions. Using this approach, we found that the 10B2 cell extracts contain approximately four times more DNA-PKcs bound to the immobilized DNA than the IRS-20 cell extracts (Fig. 3C). By manipulating the amount of DNA-PKcs in the kinase reactions this way, we found we could detect RPA kinase activity in reactions containing the equivalent of 3 μl of 10B2 cell extract (15 μg of total protein) (Fig. 3D, lanes a-c). In contrast, we could not detect any RPA kinase activity in the kinase reaction performed in parallel using 50 μl of IRS-20 cell extract containing 250 μg of total protein (Fig. 3D, lane d). These results indicate that the sensitivity of the RPA kinase assay is sufficient to detect the kinase activity of DNA-PK levels equal to and lower than that found in the IRS-20 extracts and support the idea that the IRS-20 DNA-PKcs has defective kinase activity.

The second cell line that was identified in the initial RPA-kinase assay, SX9, was found to have a defect similar to that seen with the IRS-20 cells. RPA kinase activity of cell extracts prepared from the SX9 cells were performed in parallel with extracts prepared from the parental cell line, SR1, and another SR1-derived DNA double-stranded break repair mutant cell line, SX10. Phosphorylation of the 32-kDa RPA subunit was detected in the SR1 and the SX10 cell extracts but was absent in the SX9 cell extract (Fig. 4A). RPA kinase activity of the SX9 extract was not affected by addition of purified human Ku but could be rescued by addition of purified DNA-PKcs (Fig. 4B, compare lanes b and d). This indicated that the lack of DNA-PK activity in these cells was also due to a defect in the expression or activity of the DNA-PKcs.

To ascertain whether the decrease in DNA-PK activity in the SX9 cell extracts was due to a defect in the expression of the SX9 DNA-PKcs, we probed protein blots of SR1, SX9, and SX10 cell extracts using DNA-PKcs antibodies. The results of this analysis indicated that abundance of the DNA-PKcs in the SX9 cells was reduced relative to both the wild-type SR1 and the SX10 mutant cells (Fig. 4C). However, much like the results obtained with the IRS-20 cells, we could readily detect the DNA-PKcs in the SX-9 cell extracts. Interestingly, the level of DNA-PKcs expressed in the SX-10 cells was slightly reduced relative to the wild-type cells.

To determine if the reduced kinase activity of the SX-9 DNA-PK was due to a defect in the assembly of the enzyme on DNA, we measured the retention of the DNA-PKcs from the SX9 DNA-PKcs, we probed protein blots of SR1, SX9, and SX10 cells using the indicated volumes of 10B2 (lanes a-c) and IRS-20 (lane d) cell extracts as described in Fig. 1C.

**Fig. 4. The SX9 DNA-PKcs has diminished protein kinase activity.** A, RPA kinase assays were performed as in Fig. 1C using extracts prepared from wild-type SR1 cells (lanes a and b) and the DNA repair mutants SX9 (lanes c and d) and SX10 (lanes e and f) cells. B, RPA kinase activity was measured as in Fig. 1C using SX9 cell extracts supplemented with 50 ng of purified human Ku70/Ku80 (lanes a and b) or 50 ng of purified human DNA-PKcs (lanes c and d). C, DNA-PKcs protein was analyzed as described in Fig. 2B using extracts prepared from SR1, SX9, and SX10 cells. The position of the DNA-PKcs is indicated by the arrow. D, DNA-bound DNA-PKcs protein was analyzed as in Fig. 3B using extracts prepared from SR1, SX9, and SX10 cells.

**DISCUSSION**

The modification of proteins by phosphorylation is a common mechanism for regulating a variety of biochemical activities in eukaryotic cells. Mammalian cell lines displaying defects in the

**Fig. 3. The IRS-20 DNA-PKcs has diminished protein kinase activity.** A, DNA-PKcs protein was measured in cell extracts prepared from CHO AA8 (lane a), V3 (lane b), 10B2 (lane c), and IRS-20 (lane d) cells as in Fig. 2B. B, the levels of DNA-bound DNA-PKcs protein (lanes a and b) and Ku70 protein (lanes c and d) subunits from 10B2 (lanes a and c) and IRS-20 (lanes b and d) cell extracts were determined by immunoblot analysis of immobilized DNA-agarose bead reactions performed as described under “Experimental Procedures.” Arrows indicate the position of the DNA-PKcs and Ku70 subunits. C, DNA-bound DNA-PKcs from 10B2 (lanes a and b) and IRS-20 (lane c) from the indicated amount of cell extracts was measured as described in Fig. 3B. D, RPA kinase activity was analyzed using the indicated volumes of 10B2 (lanes a-c) and IRS-20 (lane d) cell extracts as described in Fig. 1C.
expression of either the catalytic or Ku80 components of DNA-PK have defects in rejoining double-stranded DNA breaks. These results have been interpreted to indicate DNA-PK functions by phosphorylating other proteins involved in these DNA strand rejoining pathways. The data presented in this paper support the hypothesis that the protein kinase activity of DNA-PK is an important component of the double-stranded break rejoining pathway by demonstrating that the DNA repair mutant cell lines IRS-20 and SX9 both express inactive forms of the DNA-PK catalytic subunit. In murine scid and CHO V3 cells, transfer of subgenomic fragments of human chromosome 8 containing the human DNA-PKcs gene rescues the DNA-repair and V(D)J defects and restores expression of a functional DNA-PK holoenzyme (4–6). Similarly, the radiosensitivity of the IRS-20 cells can also be rescued by human chromosome 8, suggesting that these cells fall into the same genetic complementation group (xrs-7) as the scid and V3 cells (31). Our analysis of the DNA-PK status of the double-stranded break repair mutant SX9 suggests that this cell line is also in the xrs-7 complementation group.

Our data, showing that the DNA-PKcs from both IRS-20 and the SX9 cells can bind to DNA but fails to function as a protein, suggests that the IRS-20 and SX-9 cells harbor mutations that disrupt the kinase activity of the DNA-PKcs. This could be mediated via changes in critical residues in the kinase active site or by mutations that disrupt the global structure of the enzyme. Such mutations might affect both the activity of the enzyme as well as its intracellular stability. Interestingly, the levels of DNA-PKcs found in both the IRS-20 and SX9 cells is reduced relative to their parental, wild-type cell lines. This also occurs in both the murine scid and CHO V3 cells where the expression of the DNA-PKcs is severely repressed relative to wild-type levels and there is no apparent DNA-PK activity (4–6). Recently, it was shown that scid mice contain a mutation in the C terminus of the DNA-PKcs gene corresponding to amino acid position 4045 that results in the introduction of a stop codon (32). This mutation may disrupt the structure of the phosphatidylinositol-3 kinase domain of DNA-PKcs, thereby preventing accessibility of the repair machinery.
Characterization of Two DNA Double-stranded Break Repair-deficient Cell Lines That Express Inactive DNA-dependent Protein Kinase Catalytic Subunits
Scott R. Peterson, Murray Stackhouse, Mary Jo Waltman, Fanqing Chen, Koki Sato and David J. Chen

J. Biol. Chem. 1997, 272:10227-10231.
doi: 10.1074/jbc.272.15.10227

Access the most updated version of this article at http://www.jbc.org/content/272/15/10227

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 36 references, 22 of which can be accessed free at http://www.jbc.org/content/272/15/10227.full.html#ref-list-1