DNA-dependent protein kinase activity is absent in *xrs-6* cells: Implications for site-specific recombination and DNA double-strand break repair

(antigen Ku)

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ABSTRACT DNA-dependent protein kinase (DNA-PK) is a nuclear serine/threonine protein kinase composed of a catalytic subunit called p350 and a DNA binding component termed Ku. Ku consists of two tightly associated polypeptides of approximately 70 kDa and 80 kDa (Ku80). An intriguing feature of DNA-PK is that it binds to DNA ends and other discontinuities in DNA and requires these structures for its activation. This suggests that DNA-PK may function in DNA repair and/or recombination. Consistent with this, Ku DNA binding activity was shown recently to be absent in extracts of hamster *xrs-6* cells, which are defective in DNA double-strand (ds) break repair and V(D)J recombination. Furthermore, *xrs-6* cells are complemented by expression of the Ku80 cDNA. To date, DNA-PK activity has been demonstrated unequivocally only in extracts of primate cells. Here, we describe an assay that can detect DNA-PK activity in extracts of mouse, hamster, *Xenopus*, and *Drosophila* cells. Using this assay, we find that *xrs-6* cells completely lack DNA-PK activity. By contrast, *xrs-6* derivatives complemented by human chromosome fragments bearing the Ku80 gene have restored both the DNA end binding and kinase activities associated with DNA-PK. Finally, we show that *xrs-6* extracts are complemented biochemically by purified Ku. Our findings indicate that the *xrs-6* defects are direct consequences of the mutation in Ku80 and implicate DNA-PK in recombination and DNA repair processes.

A protein kinase that appears to be well suited to controlling transcription and other nuclear events is DNA-dependent protein kinase (DNA-PK), an abundant enzyme in human cells that is located primarily within the nucleus (1–5). An important feature of DNA-PK is that it interacts with DNA and must be DNA-bound to be active. We and others (6–8) have shown that DNA-PK consists of two components. One, the catalytic component, is a protein of ≈ 350 kDa (p350). On its own, however, p350 is inactive and requires the second DNA-PK component to target it to DNA and trigger its kinase activity. This second component is human autoimmune antigen Ku, which is composed of two tightly associated polypeptides of approximately 70 and 80 kDa (Ku70 and Ku80, respectively).

Although *in vivo* targets for DNA-PK have not yet been defined unequivocally, many transcription factors, including Sp1, c-Jun, c-Fos, p53, and CTF1/NF- κ B are phosphorylated by this enzyme *in vitro* (2, 3, 7, 9–11). One function of DNA-PK may therefore be to control gene expression. However, since transcription factors can also influence nuclear events such as DNA replication, recombination, and DNA repair (12–15),

these processes might also be affected by DNA-PK-mediated phosphorylation.

We and others (16–18) have shown recently that Ku DNA binding activity is absent in the Chinese hamster ovary cell line *xrs-6*, which is impaired in DNA double-strand break repair and V(D)J recombination (19–21). The Ku80 gene maps to the region of human chromosome 2 containing the gene *XRCC5*, which complements the *xrs-6* mutation (22–24). Furthermore, we have demonstrated that *xrs-6* cells can be complemented by expression of the Ku80 cDNA, indicating that *XRCC5* encodes Ku80 (18). An involvement of DNA-PK in DNA double-strand break repair would be consistent with the fact that this enzyme requires DNA termini for its activation *in vitro* (1, 2, 4, 8, 25, 26). We therefore wished to determine whether the kinase activity associated with DNA-PK is also impaired in *xrs-6* cells. Here, we develop an assay scheme that can detect DNA-PK activity in extracts of rodent, *Xenopus*, and *Drosophila* cells and use this to determine DNA-PK levels in *xrs-6* cell extracts. The results of these studies suggest that the DNA double-strand break repair and recombination defects of *xrs-6* cells may be due not only to the loss of Ku DNA end binding activity but also to the concomitant loss of DNA-PK catalytic function.

MATERIALS AND METHODS

Cells, Extract Preparation, and Protein Purification. Mammalian cells, *Xenopus laevis* cell line XL-177, and their propagation were as described (22, 27). Extracts were prepared by a modification of the method of Scholer *et al.* (28): frozen cell pellets ($0.5\text{--}3 \times 10^7$ cells) were resuspended in 100 μ l of extraction buffer [50 mM NaF, 20 mM Hepes (pH 7.8), 450 mM NaCl, 25% (vol/vol) glycerol, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, leupeptin (0.5 μ g/ml), protease inhibitor (0.5 μ g/ml), trypsin inhibitor (1.0 μ g/ml), aprotinin (0.5 μ g/ml), bestatin (40 μ g/ml)] and then frozen on dry ice and thawed at 30°C three times. After microcentrifugation for 7 min at 4°C, supernatants were stored at -70°C . Ku was purified essentially as described (6).

DNA-PK Assays. Standard peptide phosphorylation assays were as described (4). DNA-PK “pull-down” kinase assays were conducted as follows. Extract was incubated with 20 μ l of preswollen double-stranded DNA (dsDNA)-cellulose (Sigma) in a total volume of 50 μ l of Z’0.05 (25 mM Hepes/KOH at pH 7.9, 50 mM KCl, 10 mM MgCl₂, 20% glycerol, 0.1% Nonidet P-40, 1 mM dithiothreitol). The dsDNA-cellulose was then washed twice with 1 ml of Z’0.05 and resuspended in 50 μ l of Z’0.05. Samples were divided into two or three aliquots, 0.5 μ l of [$\gamma\text{-}^{32}\text{P}$]ATP (300 Ci/mmol; 1 Ci = 37 GBq) was

added, and kinase assays were conducted in the presence or absence of 4 nmol of peptide (0.2 mM). Reactions were then stopped and analyzed by spotting onto phosphocellulose paper, washing, and subjecting to liquid scintillation counting as described (4). The sequences of wild-type (wt) and mutant p53 peptides are EPPLSQEAFADLLKK and EPPLSE-QAFADLLKK, respectively. All assays were performed multiple times with at least two different extract preparations. Reproducibility of DNA-PK pull-down peptide assays (cpm incorporated for a given extract) is generally less than $\pm 10\%$.

RESULTS

Standard DNA-PK Assays Do Not Detect DNA-PK in Rodent Cell Extracts. As Ku has been reported to be a component of DNA-PK, we wished to test whether DNA-PK activity is defective in *xrs-6* cell extracts. The most sensitive DNA-PK assay hitherto described uses a synthetic peptide derived from the N-terminal transcriptional activation region of murine p53 (4). In this assay, the peptide is incubated with DNA-PK and [γ - 32 P]ATP, adsorbed onto derivatized paper, and peptide phosphorylation is determined by liquid scintillation counting after washing away unbound material. Generally, reactions are performed both in the absence and presence of DNA. Since DNA-PK appears to be the only DNA-activated kinase in mammalian cells (2, 4), the difference of these two values provides a quantitative estimate of DNA-PK activity. As shown in Fig. 1, using this method, DNA-activated peptide phosphorylation is detected readily using whole cell extract (WCE) from human 1BR cells. This kinase activity is indeed DNA-PK, because no DNA-activated phosphorylation is observed with a mutated peptide (MUT PEP) in which the DNA-PK phosphorylation site *Ser*-Gln is mutated to the sequence *Ser*-Glu, which is not recognized by DNA-PK (4, 11). Consistent with previous reports (4), this standard assay does not detect DNA-PK activity in extracts of hamster or mouse cells (Fig. 1).

Development of a DNA-PK Pull-down Peptide Assay. We considered it likely that DNA-PK would be present in rodents,

FIG. 1. Standard peptide assays detect DNA-PK in extracts of human but not rodent cells. WCE derived from human 1BR, hamster K1, or mouse 3T3 (10 μ g, 40 μ g, and 40 μ g, respectively) cells was incubated with wt peptide (wt PEP) or mutant p53 peptide (MUT PEP) as indicated, in the presence of [γ - 32 P]ATP, and the amount of peptide phosphorylation was determined. Reactions were conducted either in the absence (-) or presence (+) of linearized plasmid DNA. cpm incorporated into peptide for a typical assay are plotted.

because Ku homologs exist in mouse (29, 30) and Western blotting suggests that p350 is present in both murine and hamster cells (T.M.G. and S.P.J., unpublished data). Possible explanations for our inability to observe DNA-PK in rodent extracts were that its activity might be too low for detection or that its actions might be reversed by phosphatases. Thus, we reasoned that fractionation of rodent extracts might reveal DNA-PK activity. We therefore devised a microscale pull-down purification scheme for DNA-PK that takes advantage of the fact that this enzyme binds to dsDNA, whereas most protein phosphatases and kinases do not. In this assay, crude cell extract is incubated with dsDNA-cellulose, and then unbound material is removed by repeated washing. Because DNA-PK retains its activity when bound to DNA-cellulose, the immobilized enzyme is assayed simply by adding buffer, p53 peptide, and [γ - 32 P]ATP. High levels of p53 peptide kinase activity are recovered from human cell extracts using this approach (Fig. 2A). Addition of more extract yields higher levels of recovered activity, and an approximately linear relationship exists between the amount of extract and kinase levels over a wide range of extract concentrations. Furthermore, the pull-down assay is effective both with crude nuclear extracts (Fig. 2A) and WCEs (Fig. 2B). Since the recovered kinase is already DNA-bound, assays cannot be performed in the absence of DNA. Nevertheless, we conclude that the activity recovered is indeed

FIG. 2. DNA-PK pull-down peptide assay. (A) Titration of HeLa extract. Increasing amounts of HeLa nuclear extract (as indicated) were incubated with dsDNA-cellulose, and bound material was collected and washed. This was then incubated with [γ - 32 P]ATP, either in the absence (-) or presence (+) of wt p53 peptide, and the amount of radiolabel incorporated was determined. (B) The kinase precipitated in the pull-down assay displays DNA-PK substrate specificity. Pull-down experiments were conducted using 25 μ g of WCE from human 1BR cells, and phosphorylation reactions were conducted either in the presence of mutant (MUT) or wt p53 peptide or in the absence of peptide (none).

FIG. 3. The DNA-PK pulldown peptide assay detects DNA-PK in rodent, *Xenopus*, and *Drosophila* extracts. (A) WCE (150 μ g) from hamster K1 or mouse 3T3 cells was used in a DNA-PK pulldown peptide phosphorylation assay with wt or mutant (MUT) p53 peptide or in the absence of peptide (-). (B) WCE (150 μ g) derived from *X. laevis* cell line XL-177 or *Drosophila* embryos was used in a DNA-PK pulldown peptide phosphorylation assay with wt or mutant p53 peptide or in the absence of peptide (-).

DNA-PK, because the mutant p53 peptide is not phosphorylated significantly (Fig. 2B).

Detection of DNA-PK Activity in Extracts of Hamster, Mouse, *Xenopus*, and *Drosophila* Cells. Having established a more sensitive assay for DNA-PK, we next analyzed extracts of rodent cells. As shown in Fig. 3A, p53 peptide kinase activity is detected reproducibly in both hamster and mouse WCEs. Furthermore, kinase activity is observed with the wt but not the mutant p53 peptide, suggesting strongly that it indeed corresponds to rodent DNA-PK. Through performing titration experiments (data not shown), we estimate that mouse and hamster cell extracts contain $\approx$ 50-fold less DNA-PK than extracts of human HeLa and 1BR cells. Interestingly, DNA-PK activity is also detected in extracts of *X. laevis* XL-177 cells and of *Drosophila melanogaster* embryos (Fig. 3B), suggesting that

DNA-PK has an important and evolutionarily conserved function throughout the eukaryotic kingdom.

DNA-PK Activity Is Absent in Extracts of *xrs-6* Cells. Since *xrs-6* cells lack Ku DNA binding activity, we used the pulldown assay to test extracts of these cells for DNA-PK. Thus, we discovered that, unlike the wt hamster cell line K1, *xrs-6* cells contain no DNA-PK activity (Fig. 4A). This deficiency appears to be linked to the DNA damage and recombination defects of *xrs-6* cells because DNA-PK activity is also undetectable in extracts of the independently derived hamster lung cell line XR-V15B, which falls into the same complementation group as *xrs-6* (Fig. 4B).

***xrs-6* Cells Complemented by Human DNA Fragments Have Restored DNA-PK Levels.** We have recently shown that the Ku80 gene corresponds to the human *XRCC5* gene (18). The

FIG. 4. *xrs-6* and XR-V15 cells lack DNA-PK. (A) K1 or *xrs-6* extract (150 μ g) was analyzed by the DNA-PK pulldown peptide assay with wt or mutant (MUT) p53 peptide or in the absence of peptide (-). (B) DNA-PK pulldown peptide assays were performed using V79 or XR-V15 cell extracts in the absence (-) or presence (+) of wt p53 peptide substrate.

Ku80 gene maps to the human chromosome region 2q33-35 (24), and we have generated a series of hamster-human cell hybrids derived from *xrs-6* cells that contain different portions of human DNA from this region (22, 23). Some hybrids, such as H22 and D2-X-38, contain the Ku80 gene and complement the *xrs-6* mutation, whereas others such as 38D do not contain an intact Ku80 gene and do not complement the mutation (18, 23). When extracts from these clones were used in DNA-PK pulldown assays, we found that complementing hybrids H22 and D2-X-38 have DNA-PK activity, whereas noncomplementing hybrid 38D lacks detectable DNA-PK (Fig. 5A). Kinase activity is consistently lower in hybrid D2-X-38 compared to hybrid H22, which correlates with the intermediate radiosensitivity of D2-X-38, which we have attributed to instability of the human DNA fragment in this line (18). These results indicate that the defect in Ku80 in *xrs-6* cells also results in a loss of DNA-PK activity.

Biochemical Complementation of *xrs-6* Extracts with Purified Ku. If the DNA-PK defect of *xrs-6* extracts is due solely

FIG. 5. (A) DNA-PK activity correlates with radiation resistance. Extracts (150 μ g), as indicated, were analyzed by the DNA-PK pulldown peptide assay in the absence (-) or presence (+) of wt p53 peptide. Cell lines employed are described in the text. (B) Biochemical complementation of the *xrs-6* DNA-PK defect. *xrs-6* extract alone (150 μ g), *xrs-6* extract (150 μ g) in the presence of 200 ng of purified Ku, or 200 ng of Ku alone was analyzed by the DNA-PK pulldown peptide assay.

to a lack of Ku, then the addition of purified Ku should restore DNA-PK activity. Previously, we have shown that p350 cannot bind DNA in the absence of Ku (8) and therefore anticipated that Ku would restore kinase activity only if added prior to the dsDNA-cellulose pulldown step. Consistent with this, when Ku is added to dsDNA-cellulose that has been preincubated with *xrs-6* extracts, no complementation is achieved (data not shown). By contrast, when a purified preparation of Ku is mixed with the *xrs-6* extract before adding the dsDNA-cellulose, DNA-PK activity is obtained (Fig. 5B). We thus conclude that *xrs-6* cells contain active p350 and that the DNA-PK defect is due to a specific deficiency in Ku.

DISCUSSION

In this paper, we describe an assay scheme for DNA-PK that employs a microscale DNA-PK pulldown purification on dsDNA-cellulose as its first step. This assay is much more sensitive than standard DNA-PK assays for several reasons. First, the pulldown step can concentrate DNA-PK from dilute samples. Second, because most kinases do not bind to dsDNA-cellulose significantly, background phosphorylation by other kinases is reduced greatly. Third, the pulldown step removes endogenous kinase substrates that contribute to the high levels of phosphorylation in standard DNA-PK assays in the absence of peptide. Finally, protein phosphatases that reverse phosphorylation by DNA-PK are removed by the pulldown step.

Using this technique, we have shown that DNA-PK exists in hamster, mouse, *Xenopus*, and *Drosophila* cells. This is consistent with the fact that Ku homologs have been identified in mouse and *Drosophila* (29-31) and suggests strongly that DNA-PK may play a role in DNA repair and site-specific recombination in diverse eukaryotes. Indeed, the recent identification of a Ku-like activity in *Saccharomyces cerevisiae* (32) suggests that Ku/DNA-PK is ubiquitous in the eukaryotic kingdom. Interestingly, we find that primate cells have 10- to 50-fold more DNA-PK activity than other species that we have examined. Although the reason for this is unclear at present, it is tempting to speculate that primate cells may be particularly effective at recognizing DNA double-strand breaks or performing certain types of site-specific recombination. When we examined extracts of *xrs-6* and XR-V15B cells, we found them to be devoid of DNA-PK activity. Since this deficiency is complemented by the addition of purified Ku, this reinforces our prior conclusion that the primary defect of *xrs-6* cells is the DNA end-binding activity associated with Ku. Indeed, we show that the kinase activity associated with the p350 component of DNA-PK is potentially functional in *xrs-6* and XR-V15B cells. Our results also demonstrate that a direct consequence of the Ku defect is a concomitant deficiency in the kinase activity of DNA-PK. These data with mutant cells thus suggest strongly that Ku/DNA-PK functions as a complex *in vivo*, as it does *in vitro*, and indicate that kinase activity requires Ku. Furthermore, our results suggest that no other endogenous proteins can target p350 to dsDNA and cooperate with p350 to yield DNA-PK activity.

xrs-6 cells are sensitive specifically to agents that induce DNA double-strand breaks (19, 20). Thus, the DNA damage sensitivity profile of *xrs-6* cells matches the fact that DNA-PK is activated *in vitro* by dsDNA ends (1, 2, 4, 8) and suggests that DNA-PK functions in DNA repair by recognizing such structures. The observation that immunoglobulin V(D)J site-specific recombination is also defective in *xrs-6* cells additionally implicates DNA-PK in this process. Consistent with this, the V(D)J recombination defect of *xrs-6* cells is not in generating site-specific DNA double-strand cuts but in subsequent steps that may overlap with the repair of dsDNA lesions that are generated by mutagenic agents (19).

Our results raise the intriguing possibility that the p350 catalytic component of DNA-PK executes important functions

in DNA repair and recombination. One way to verify this will be to determine whether mutants defective in p350 are impaired in DNA double-strand break repair. It will clearly be of great interest to determine whether p350 is defective in cell lines such as XR-1 and V3/*scid*, which are impaired in DNA double-strand break repair and V(D)J recombination but belong to different complementation groups from *xrs-6* (refs. 33–35 and references therein). Unfortunately, the gene encoding p350 has not yet been cloned fully, and this is proving to be an arduous task since it is a large gene, which spans more than 100 kb of genomic DNA.

There are several mechanisms (not necessarily mutually exclusive) by which DNA-PK may function in DNA repair and recombination. One is that the binding of Ku/DNA-PK to DNA termini protects them from nuclease digestion or assists in the alignment of two adjacent broken ends. In line with a role in protecting DNA ends from nucleases, the rare V(D)J recombination products that are generated in *xrs-6* cells frequently bear large deletions, presumably as a result of nuclease attack (21). Such DNA end protection could just require Ku but might also require the entire DNA-PK complex containing p350. Another possibility is that, when bound to DNA ends, DNA-PK phosphorylates and activates components of the DNA repair and recombination apparatus and thus restricts their activities to the appropriate cellular location. A third possibility is that DNA-PK enhances repair indirectly by phosphorylating and inactivating transcription factors to allow access by the repair and recombination machinery. Finally, it is possible that DNA-PK functions as a DNA-end sensor and that its activation initiates a signaling pathway to alert the cell to the fact that it has sustained DNA damage (36). The availability of cell lines deficient in DNA-PK should prove extremely useful in examining these exciting possibilities.

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