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DNA-Dependent Protein Kinase Catalytic Subunit: A Relative of Phosphatidylinositol 3-Kinase and the Ataxia Telangiectasia Gene Product

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Summary

DNA-dependent protein kinase (DNA-PK), which is involved in DNA double-stranded break repair and V(D)J recombination, comprises a DNA-targeting component called Ku and an ~460 kDa catalytic subunit, DNA-PK$_{cs}$. Here, we describe the cloning of the DNA-PK$_{cs}$ cDNA and show that DNA-PK$_{cs}$ falls into the phosphatidylinositol (PI) 3-kinase family. Biochemical assays, however, indicate that DNA-PK phosphorylates proteins but has no detectable activity toward lipids. Strikingly, DNA-PK$_{cs}$ is most similar to PI kinase family members involved in cell cycle control, DNA repair, and DNA damage responses. These include the FKBP12-rapamycin-binding proteins Tor1p, Tor2p, and FRAP, S. pombe rad3, and the product of the ataxia telangiectasia gene, mutations in which lead to genomic instability and predisposition to cancer. The relationship of these proteins to DNA-PK$_{cs}$ provides important clues to their mechanisms of action.

Introduction

DNA-dependent protein kinase (DNA-PK) is a nuclear protein Ser/Thr kinase present in a wide range of eukaryotic species (Walker et al., 1985; Anderson and Lees-Miller, 1992; Finnie et al., 1995). A conspicuous feature of DNA-PK is that it must be DNA bound to express its catalytic properties. DNA-PK can be fractionated into two components (Gottlieb and Jackson, 1993; Dvir et al., 1992). One is a large polypeptide corresponding to the DNA-PK catalytic subunit (DNA-PK$_{cs}$). On its own, DNA-PK$_{cs}$ is inactive and relies on the other DNA-PK component to direct it to the DNA and trigger its kinase activity. This second component is autoimmune antigen Ku, comprising polypeptides of approximately 70 kDa and 80 kDa in a heterodimeric complex. One physiological function for DNA-PK may be to modulate transcription, since it phosphorylates several transcription factors in vitro (for review see Anderson and Lees-Miller, 1992) and is a potent inhibitor of transcription by RNA polymerase I (Kuhn et al., 1995; Labhart, 1995).

DNA-PK activation requires DNA double-stranded breaks (DSBs) or other discontinuities in the DNA double helix, owing to the fact that Ku binds specifically to these structures (Gottlieb and Jackson, 1993; Morozov et al., 1994). This suggests that DNA-PK may function in vivo by recognizing DNA ends at sites of DNA damage or that occur as recombination intermediates. Indeed, recent work has revealed that cells defective in DNA-PK components are hypersensitive to killing by ionizing radiation owing to an inability to repair DSBs effectively (Jeggo et al., 1995; Jackson and Jeggo, 1995; Roth et al., 1995, and references therein). Cells defective in either Ku or DNA-PK$_{cs}$ are also unable to perform V(D)J recombination, the site-specific recombination process that takes place in developing B and T lymphocytes to generate the variable regions of immunoglobulin and T cell receptor genes. In the absence of DNA-PK function, V(D)J recombination intermediates are unable to be processed and ligated. The DNA-PK holoenzyme is therefore a crucial component of the DNA DSB repair and V(D)J recombination apparatus.

To understand fully the structure and mechanism of action of DNA-PK$_{cs}$, it is necessary to obtain and characterize its cDNA. Here, we describe the isolation of the DNA-PK$_{cs}$ cDNA. Surprisingly, analysis of the DNA-PK$_{cs}$ sequence reveals homology to the catalytic domains of mammalian phosphatidylinositol 3-kinase (PI3K) and its relatives. Some members of the PI3K family, such as mammalian PI3K itself, function in signal transduction by phosphorylating inositol phospholipids, which serve as intracellular second messengers (for review see Kapeller and Cantley, 1994). Other kinases in this group are implicated in processes ranging from controlling membrane traffic and regulating nuclear envelope assembly to modulating cell cycle progression and functioning in DNA repair and DNA damage–sensing pathways (for example, Herman et al., 1992; Kapeller and Cantley, 1994; Kato and Ogawa, 1994; Kunz et al., 1993; Brown et al., 1994; Savitsky et al., 1995). Because DNA-PK$_{cs}$ falls into the PI3K family, we have investigated whether DNA-PK can utilize phosphatidylinositides as substrates. The results of these studies are discussed in regard to DNA-PK function and with reference to the physiological roles and mechanisms of action of other members of the PI3K superfamily.

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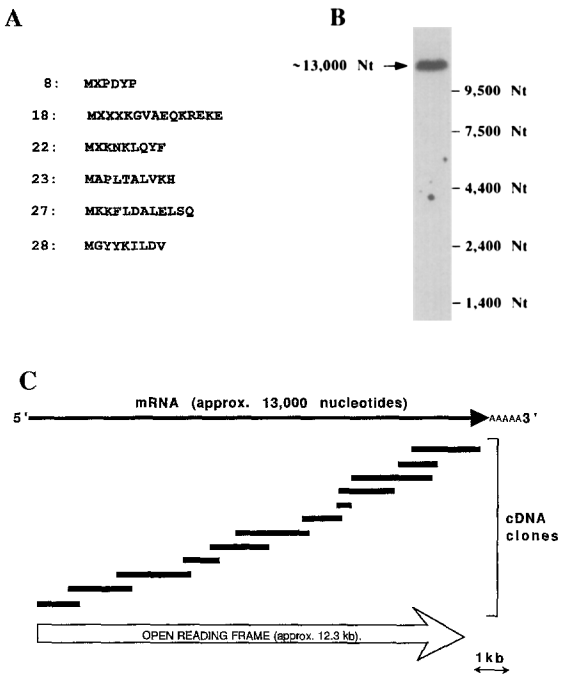

Figure 1. Isolation of DNA-PK$_{cs}$ cDNA Clones

(A) Partial sequences of peptides derived from DNA-PK$_{cs}$. X indicates positions where amino acid assignment was ambiguous.

(B) DNA-PK$_{cs}$ cDNA clone N1 hybridizes to a large mRNA. HeLa poly(A)$^+$ RNA was electrophoresed on a 0.8% agarose gel and subjected to Northern blot transfer and hybridization analysis using the clone N1 insert as probe.

(C) Schematic representation of the DNA-PK$_{cs}$ mRNA, the set of overlapping cDNA clones that span the cDNA, and the location of the DNA-PK$_{cs}$ ORF.

Results

Isolation of a Partial DNA-PK$_{cs}$ cDNA Clone

To obtain DNA-PK$_{cs}$ cDNA clones, we cleaved purified DNA-PK$_{cs}$ by treatment with cyanogen bromide, resolved the resulting mixture of peptides by reverse-phase high pressure liquid chromatography, and determined the partial sequences of several peptides (Figure 1A). Oligonucleotides corresponding to these sequences were then generated and used to screen a random-primed λgt10 HeLa cDNA library. In the case of the oligonucleotide corresponding to peptide 18, a positively hybridizing plaque was identified, and the cDNA insert contained within the λ bacteriophage was isolated and cloned into a plasmid vector. Sequencing of this clone, termed N1, revealed the presence of an open reading frame (ORF) that contains the sequence of peptide 18. Consistent with the large size of DNA-PK$_{cs}$, the cDNA fragment of clone N1 recognizes an RNA species of over 12 kb in Northern blot hybridization assays (Figure 1B). RNase H mapping experiments using oligonucleotides derived from clone N1 (data not shown) indicate that the middle of this cDNA fragment lies approximately 9 kb from the 5′ end and 5 kb away from the 3′ end of the mRNA species to which it hybridizes.

Isolation and Characterization of Clones Spanning the DNA-PK$_{cs}$ cDNA

Using the clone N1 insert as a probe, clones were isolated that extend in the 5′ (upstream) or 3′ (downstream) directions of the putative DNA-PK$_{cs}$ mRNA. By repeating this procedure, a set of clones spanning approximately 13 kb was obtained (Figure 1C). In every case tested, including the clone at the 5′ and 3′ ends of the set, the inserts were found to recognize the same large mRNA species detected by clone N1 (data not shown). In line with the large size of DNA-PK$_{cs}$, the compiled sequence contains an ORF of 12,228 bp, extending from the beginning of the sequence to the UGA termination codon 1664 nt upstream from the poly(A) segment at the end of the 3′-most clone. The sequence 3′ to the UGA codon has multiple stop codons in all three frames, indicating that the carboxyl terminus of the encoded protein has been reached. A poly(A) addition signal (AAUAAA) is present 16 nt before the poly(A) segment, suggesting that the 3′ end of the cDNA is complete.

The fact that the ORF begins at the 5′ end of the cloned sequence suggests that the extreme 5′ end of the cDNA has not been reached. However, we have been unable to isolate additional 5′ sequences from several cDNA libraries. Nevertheless, we tentatively identify methionine residue, denoted amino acid 1 in Figure 2, as the start site for translation. This is the first methionine codon in the ORF, and its context is consistent with its use as an efficient start site for protein synthesis (Kozak, 1991). In line with primer extension analyses, a possible transcription initiation site is present approximately 50 nt upstream of the putative DNA-PK$_{cs}$ translation initiation codon in the sequence of a corresponding genomic clone (M. A. C. et al., unpublished data). The ORF in the genomic sequence ends just 5′ to the putative transcription start site, and no methionine codons are found between this site and the methionine codon we have denoted codon 1. Importantly, the predicted DNA-PK$_{cs}$ sequence (Figure 2) contains all of the DNA-PK$_{cs}$ peptide sequences that we have determined. Assuming that our assignment of the initiator methionine is correct, the nascent DNA-PK$_{cs}$ polypeptide contains 4,096 amino acid residues and has a predicted molecular mass of 465,482 Da. This is considerably larger than the size of DNA-PK$_{cs}$ estimated previously by SDS–polyacrylamide gel electrophoresis, presumably reflecting large inaccuracies in determining the size of this very large polypeptide.

To verify that the composite cDNA indeed encodes DNA-PK$_{cs}$, we overexpressed portions of the ORF in Escherichia coli, purified the encoded polypeptides, and then used these as antigens to raise antisera in rabbits. As shown in Figure 3A, antiserum raised against the protein product of clone N1 specifically recognizes DNA-PK$_{cs}$ in Western blots of crude HeLa cell nuclear extracts and in purified preparations of DNA-PK. In contrast, preimmune sera do not detect this protein. Furthermore, the anti-clone N1 antiserum immunoprecipitates a large polypeptide from extracts of [^{35}S]methionine-labeled HeLa cells, whereas

Figure 2. Sequence of the DNA-PK$_{cs}$ Polypeptide

Translation is assumed to initiate at the ATG methionine codon denoted 1, as its context (CGGCGGCATGG) conforms well to the translation initiation site consensus (Kozak, 1991; see text). The locations of DNA-PK$_{cs}$ peptide sequences (Figure 1A) are indicated by underlining, and potential DNA-PK autophosphorylation sites (SQ and TQ) are in bold. Leucine residues forming part of the leucine zipper motif are indicated by asterisks. The carboxy-terminal region with homology to other kinases (see Figure 4) is also underlined.

the preimmune control serum does not (Figure 3B). Antisera raised against other portions of the DNA-PK$_{cs}$ ORF also detect DNA-PK$_{cs}$ in Western blotting and immunoprecipitation studies (data not shown).

To confirm that the polypeptide recognized by antibodies raised against the product of clone N1 is indeed DNA-PK$_{cs}$, we employed monoclonal antibody 42-27, which had been raised against purified human DNA-PK$_{cs}$ and shown to immunoprecipitate DNA-PK activity (Carter et al., 1990). Significantly, antibody 42-27 recognizes the >400 kDa polypeptide immunoprecipitated by the anti–clone N1 product antiserum (Figure 3C). As further verification that the composite cDNA encodes DNA-PK$_{cs}$, monoclonal antibodies 42-27, 25-4, and 18-2 raised against purified human DNA-PK$_{cs}$ (Carter et al., 1990) specifically recognize portions of the DNA-PK$_{cs}$ polypeptide overexpressed in E. coli (data not shown). Finally, these monoclonal and polyclonal sera raised against purified human DNA-PK$_{cs}$ recognize a polypeptide of the same size as DNA-PK$_{cs}$ that is encoded by yeast artificial chromosomes that we have identified by hybridization to the cloned cDNA segments. These yeast artificial chromosomes complement the DNA repair and V(D)J recombination defects of hamster V3 cells, which lack functional DNA-PK$_{cs}$ (Blunt et al., 1995).

Analysis of the DNA-PK$_{cs}$ Protein Sequence

Examination of the DNA-PK$_{cs}$ sequence reveals that the polypeptide has a predicted isoelectric point value of 6.9 and its amino acid distribution is consistent with it being a soluble protein. Although the amino-terminal approximately 400 kDa of DNA-PK$_{cs}$ is not significantly homologous to other known proteins, the region comprising the carboxy-terminal approximately 380 amino acid residues is highly related to the carboxy-terminal catalytic domains of proteins falling into the PI3K superfamily (Figure 4). Although they are clearly distinct from classical protein kinases, it is noteworthy that DNA-PK$_{cs}$ and other PI3K family members contain the motifs DXXXXN and DFG, which in conventional protein kinases play critical roles in catalysis (Taylor et al., 1992).

From the multiple sequence alignment in Figure 4, it is clear that PI3K family members fall into two distinct

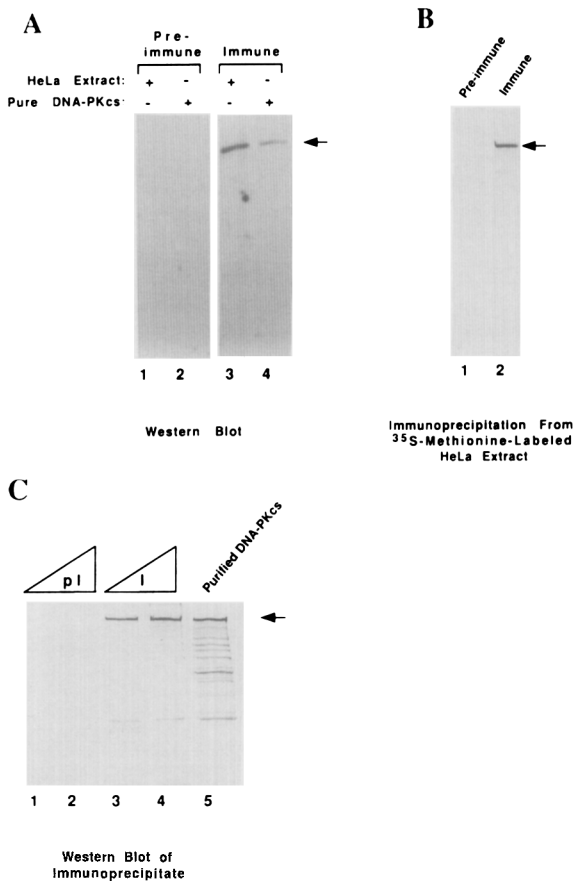

Figure 3. DNA-PK$_{cs}$ Is Detected by Antisera Raised Against the Protein Product of the Clone N1 ORF

(A) Anti–clone N1 antisera detect DNA-PK$_{cs}$ in crude HeLa nuclear extracts. Nuclear extract (4 μg; lanes 1 and 3) or purified DNA-PK$_{cs}$ (20 ng; lanes 2 and 4) were electrophoresed on a 5% SDS–polyacrylamide gel and subjected to Western immunoblot analysis. Lanes 1 and 2 were probed with preimmune serum and lanes 3 and 4 were probed with anti–clone N1 antiserum.

(B) Anti–clone N1 antiserum immunoprecipitates a >350 kDa polypeptide from extracts of HeLa cells grown in the presence of [^{35}S]methionine. Extracts were incubated with preimmune serum (lane 1) or anti–clone N1 antiserum (lane 2), and then immunoprecipitates were collected and analyzed by SDS–polyacrylamide gel electrophoresis and autoradiography.

(C) The polypeptide immunoprecipitated by anti–clone N1 antiserum is recognized by a monoclonal antibody raised against DNA-PK$_{cs}$. Immunoprecipitations using preimmune (pI) or anti–clone N1 antiserum (I) were performed using 10 μg of nonradiolabeled HeLa cell nuclear extract, and then samples were electrophoresed on a 5% SDS–polyacrylamide gel and subjected to Western immunoblot analysis using anti-DNA-PK$_{cs}$ monoclonal antibody 42-27. Lane 5 contains 20 ng of purified DNA-PK$_{cs}$ as a control.

subgroups. Subgroup 1 contains proteins such as the 110 kDa (p110) subunit of mammalian PI3K, Saccharomyces cerevisiae Vps34p, and the yeast PI 4-kinase (PI4K), Pik1p. Subgroup 2 contains DNA-PK$_{cs}$, S. cerevisiae Tor1p and Tor2p (also referred to as Drr1p and Drr2p, respectively), and their mammalian homolog FRAP (additionally named RAFT1 and RAPT1). Also in subgroup 2 are the product of the ataxia telangiectasia (AT) gene, ATM, Schizosaccharomyces pombe rad3 (the carboxy-terminal kinase domain of rad3 was not recognized originally by Seaton et al. [1992] owing to sequencing errors; A. M. Carr, personal communication), and its S. cerevisiae homolog Esr1p (which is identical to Mec1p; T. Weinert personal communication). There are several criteria by which the two subgroups can be distinguished. First, members of a subgroup are more related to one another than to members of the other subgroup. Second, members of a subgroup share similar spacing between the regions of greatest homology within the kinase domain. Third, members of subgroup 2 such as DNA-PK$_{cs}$ have in common a region of homology at their extreme carboxyl terminus that is not present in proteins of subgroup 1. Finally, members of the DNA-PK$_{cs}$ subgroup are all over 270 kDa in size, whereas those of the other subgroup range from 95–110 kDa.

Outside its carboxy-terminal region, DNA-PK$_{cs}$ is not significantly related to other known proteins, and no obvious repeating motifs are evident. However, it is noteworthy that DNA-PK$_{cs}$ contains Ser–Gln motifs (underlined in Figure 2B), which are consensus DNA-PK phosphorylation sites (Bannister et al., 1993; Anderson and Lees-Miller, 1992). As DNA-PK can autophosphorylate, some of the Ser–Gln motifs may be employed in autoregulation of DNA-PK activity. It is also of interest to note that DNA-PK$_{cs}$ possesses a "leucine zipper" motif that may potentially direct interactions with Ku or other proteins.

DNA-PK$_{cs}$ Does Not Appear to Phosphorylate Lipids

Since a potent inhibitor of mammalian PI3K is the sterol-like anti-fungal compound wortmannin, we tested whether wortmannin could inhibit protein phosphorylation by purified DNA-PK. As shown in Figure 5, phosphorylation of transcription factor Sp1 by DNA-PK is essentially abolished by 500 nM wortmannin. Titration studies using both Sp1 (Figure 5) and synthetic peptides (data not shown) as DNA-PK substrates indicate that the wortmannin concentration required for half-maximal inhibition is around 250 nM. This compares with a figure of 5 nM for mammalian PI3K (Okada et al., 1994).

To see whether DNA-PK is a PI kinase, we tested DNA-PK preparations derived from HeLa cell nuclei for an ability to phosphorylate lipids, including diacylglycerol, PI, PI (4)phosphate, and PI (4,5)bisphosphate. In these studies, we were unable to detect lipid kinase function other than low amounts of PI4K activity (Figure 5C). Significantly, this PI4K activity is not stimulated by DNA. To ascertain whether the weak PI4K activity is a property of DNA-PK$_{cs}$, we purified DNA-PK further by chromatography on heparin–agarose. Notably, although this fractionation scheme recovers high levels of protein kinase activity (Figure 5B), only trace PI4K activity is detectable in the resulting highly purified DNA-PK preparation (Figure 5C). This suggests strongly that DNA-PK is not a PI4K. Consistent with this, wortmannin has no effect on the weak PI4K activity detected in DNA-PK preparations derived from HeLa nuclear extracts (data not shown).

To exclude the possibility that Ku inhibits a lipid kinase activity associated with DNA-PK$_{cs}$, we separated the two DNA-PK components by chromatography. The resulting essentially homogeneous DNA-PK$_{cs}$ preparation was still active in its ability to phosphorylate proteins when ana-

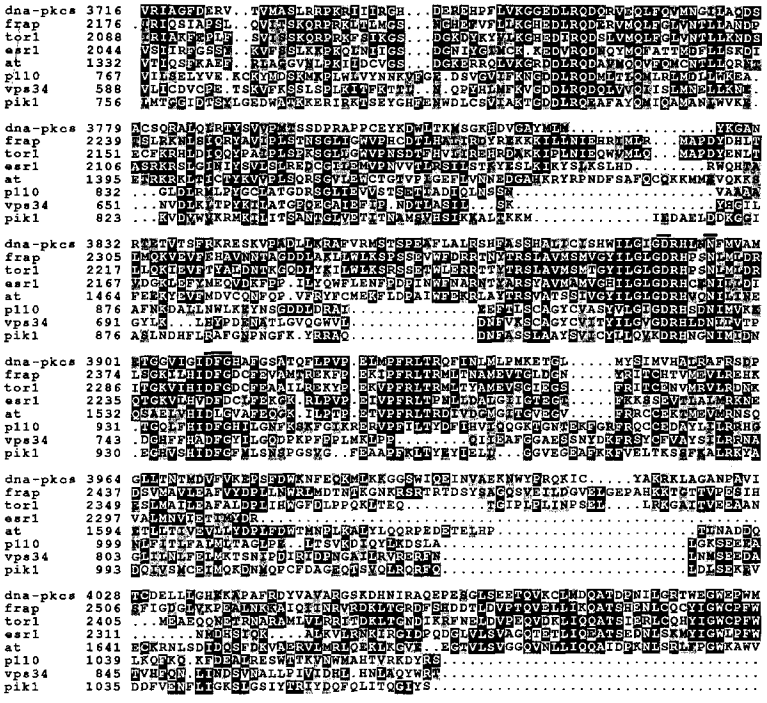

Figure 4. Sequence Alignment between the Carboxy-Terminal Regions of Various Members of the PI3K Superfamily

Black background indicates identity and stippling indicates similarity across the members of the PIK family. The motifs DXXXXN and DFG, which by analogy with protein Ser/Thr kinases are believed to play important roles in catalysis, are overlined in the DNA-PK$_{cs}$ sequence. Abbreviations used and GenBank data base accession numbers are as follows: dna-pkcs, DNA-PK$_{cs}$ (U34994); frap, human FRAP (L34075); tor1, S. cerevisiae Tor1p (P35169); esr1, S. cerevisiae Esr1p (P38111); at, the product of the *ATM* gene (U26455); p110, the 110 kDa catalytic subunit of human PI3K (S67334); vps34, S. cerevisiae Vps34p (P22543); pik1, S. cerevisiae PI4K Pik1p (P39104).

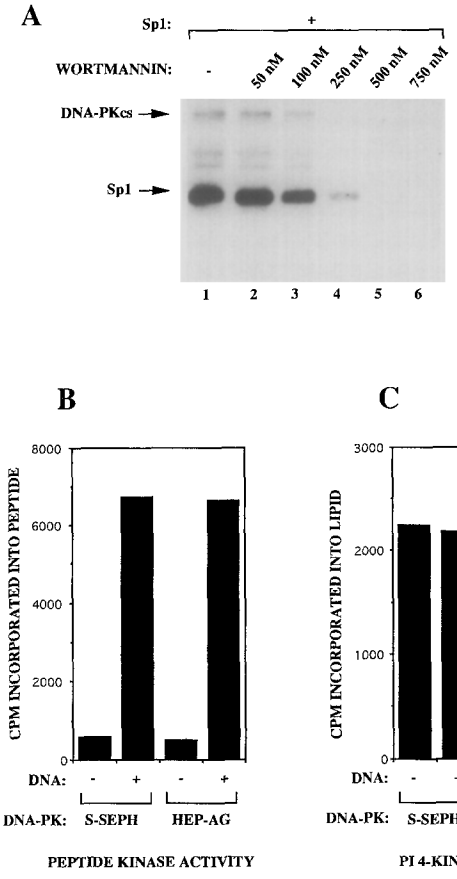

Figure 5. Testing DNA-PK Derived from HeLa Cell Nuclei for Lipid Kinase Function

(A) Wortmannin inhibits DNA-PK activity. DNA-PK (0.5 μl) was incubated with 100 ng of Sp1 and 5 μCi of [γ-^{32}P]ATP (3000 Ci/mmol) as described in Experimental Procedures. Assays were conducted in the absence of wortmannin (lane 1) or in the presence of 50 nM (lane 2), 100 nM (lane 3), 250 nM (lane 4), 500 nM (lane 5), or 750 nM (lane 6) wortmannin. Samples were analyzed by electrophoresis on a 9% SDS–polyacrylamide gel, followed by autoradiography. The positions of the Sp1 and DNA-PK$_{cs}$ are indicated. Other radiolabeled bands are DNA-PK$_{cs}$ degradation products.

(B and C) Although weak PI4K activity is present in partially purified DNA-PK preparations (the last purification step is S–Sepharose [S-SEPH]), this activity does not copurify further with DNA-PK upon chromatography on heparin–agarose (HEP-AG).

lyzed in the presence of purified Ku, but lacked detectable lipid kinase activity (data not shown). The above assays were conducted in the presence of Mg^{2+}. Since Mn^{2+} is required for the p110 subunit of mammalian PI3K to phosphorylate its 85 kDa regulatory subunit effectively (Carpenter et al., 1993), we performed assays in the presence of this cation. Again, no DNA-PK-associated lipid kinase activities were detected. We were also unable to detect DNA-PK-mediated lipid phosphorylation when we presented potential substrates together with other lipids that are compositionally similar to a cell membrane. All lipid kinase assays were conducted under conditions in which DNA-PK was capable of recognizing peptide substrates and control lipid kinases were highly active. In conclusion, despite testing DNA-PK at various stages of purification under a wide variety of assay conditions, both in the presence and absence of DNA and in the absence and presence of Ku, we have been unable to detect phosphorylation of any PI derivative that is available.

Discussion

DNA-PK$_{cs}$ Is a Very Large Protein

In this manuscript, we describe the isolation of a series of overlapping cDNA clones encoding an approximately 460 kDa polypeptide that corresponds to DNA-PK$_{cs}$. The large size of DNA-PK$_{cs}$ suggests that it contains multiple functional domains. DNA-PK-mediated protein phosphorylation is presumably conducted by the region homologous to the catalytic domains of PI3K family members. However, this remains to be established unequivocally, as we have been unable to obtain kinase activity by overexpressing recombinant carboxy-terminal derivatives of DNA-PK$_{cs}$ in E. coli (derivatives analyzed include those spanning residues 3565–4096, 3565–4005, 3786–4096, 3734–4096, 3591–4061, 3591–4096, and 3734–4061 and have been tested both in the presence and absence of Ku for Sp1 and peptide phosphorylation activity). Other regions of DNA-PK$_{cs}$ are likely to mediate interactions with Ku and DNA. It is doubtful, however, that the whole DNA-PK$_{cs}$ polypeptide is dedicated to the above activities, and it is probable that DNA-PK$_{cs}$ also performs other functions. Although no homologies have been detected between DNA-PK$_{cs}$ and DNA-modifying enzymes, it is possible that DNA-PK$_{cs}$ catalyzes alterations of the DNA template to promote DNA repair. Instead, or in addition, DNA-PK$_{cs}$ might recruit the other repair/recombination proteins to the site of the DNA DSB. Candidates for interacting proteins include the V(D)J recombination activating proteins RAG1 and RAG2, the product of the *XRCC4* gene, which complements the DSB repair–defective XR-1 cell line, DNA ligases, and enzymes that function in V(D)J recombination and possibly DNA DSB repair by modifying DNA ends before they are joined (for more detailed discussions of DNA-PK function, see Jeggo et al., 1995; Jackson and Jeggo, 1995; Roth et al., 1995).

DNA-PK$_{cs}$ Is Homologous to Lipid Kinases, but Functions as a Protein Kinase

Since DNA-PK$_{cs}$ is homologous to the catalytic domains of proteins that phosphorylate PI and its derivatives, it is conceivable that DNA-PK generates phosphorylated lipids in response to DNA damage and that these molecules serve as intracellular second messengers to elicit changes in cellular metabolism or cell cycle progression to facilitate DNA repair. We have not, however, been able to detect lipid phosphorylation by DNA-PK using an array of potential substrates and assay conditions, suggesting that DNA-PK is not, in fact, a lipid kinase. Nevertheless, it is possible that DNA-PK phosphorylates lipids other than those employed in our experiments, functions as a lipid kinase under different assay conditions, or requires association with another polypeptide to display lipid phosphorylation activity.

Although the protein kinase activity of DNA-PK conflicts with the prevailing opinion on the mechanisms by which PI3K family members act, it is now established that even some bona fide lipid kinases can phosphorylate proteins. Thus, yeast Vps34p is capable of autophosphorylation (Stack and Emr, 1994) and the p110 subunit of mammalian PI3K phosphorylates its associated 85 kDa (p85) regulatory component (for example, Carpenter et al., 1993; Dhand et al., 1994). Notably, only associated regulatory subunits are phosphorylated effectively by p110, suggesting that p110 relies on close proximity to its substrate. DNA-PK also appears to require close juxtaposition of the target protein, since it works effectively only when bound to the same DNA molecule as its substrate (Gottlieb and Jackson, 1993). Another parallel between DNA-PK$_{cs}$ and p110 is the use of regulatory components. In mammalian PI3K, p110 associates with p85, which physically links the kinase to activated growth factor receptors. Similarly, DNA-PK$_{cs}$ is targeted to DNA through Ku serving as an adaptor. When DNA-PK and PI3K are better understood, it will be of interest to see whether there are similarities in their activation mechanisms.

Relationship of DNA-PK to Other PI Kinase Family Members Involved in Controlling Cell Cycle Progression, DNA Repair, and the Maintenance of Genomic Integrity

The DNA-PK$_{cs}$ subgroup of the PI3K superfamily includes Tor1p and Tor2p of S. cerevisiae and their mammalian homolog FRAP. These proteins control advancement through the cell cycle and are downstream targets for the immunosuppressant rapamycin, which effects arrest at the G1–S cell cycle transition (for example, Kunz et al., 1993; Brown et al., 1994). Another protein in the DNA-PK$_{cs}$ subgroup is S. pombe rad3 and its S. cerevisiae homolog, Esr1p (Seaton et al., 1992; Kato and Ogawa, 1994). Mutations in rad3 or Esr1p result in an inability to perform meiosis, an inability to maintain the dependence of mitosis on the completion of DNA synthesis, and sensitivity to ionizing radiation and ultraviolet light (Al-Khodairy and Carr, 1992; Jimenez et al., 1992; Seaton et al., 1992; Kato and Ogawa, 1994; Weinert et al., 1994). A third protein of the DNA-PK$_{cs}$ subgroup is ATM, the product of the gene that is mutated in the human autosomal recessive disorder AT (Savitsky et al., 1995, and references therein). AT is characterized by an array of symptoms, including cerebellar degeneration, dilated blood vessels, growth retardation, immune deficiency, premature aging, chromosomal instability, and predisposition to cancer. At the cellular level, AT cells are notable in that they are hypersensitive to ionizing radiation, they are impaired in mediating the inhibition of semiconservative DNA synthesis and the G1–S and G2–M cell cycle checkpoints in response to ionizing radiation, and they display delays in p53 induction after irradiation.

To our knowledge, lipid phosphorylation activities have not been ascribed to any of the proteins falling into the DNA-PK$_{cs}$ subgroup of the PI3K family. Given our findings regarding the catalytic properties of DNA-PK, we suggest that, instead of or in addition to functioning through phospholipid signaling pathways, Tor1p, Tor2p, FRAP, rad3/Esr1p, and ATM serve as protein kinases. Although there are many possible targets for such kinases, it is interesting to note that TORs/FRAP, rad3/Esr1p, and ATM all regulate cell cycle progression, presumably by influencing the activity of p34^{cdc2} and its relatives. It may therefore be that

these proteins feed into protein phosphorylation cascades that modulate the activity of the cell cycle machinery.

Like DNA-PK, ATM and rad3/Esr1p are intimately linked to DNA repair and DNA-damage recognition. It is therefore appealing to propose that these proteins function in distinct but analogous pathways for sensing and responding to aberrations in genomic integrity (it is possible that the TOR family of proteins sense the integrity of some other cellular component). Thus, rad3/Esr1p and ATM might be activated by damaged or partially replicated DNA in a manner that resembles the activation of DNA-PK by DNA DSBs. A corollary of this model is that rad3 and ATM will interact with Ku-like adaptor components. There are several ways in which DNA-PK, ATM, and rad3 could operate. One is that the activation of these kinases by DNA damage triggers processes that prevent cell cycle progression. Consistent with this, cells mutant for ATM or rad3 do have checkpoint defects. Although no clear checkpoint aberrations have been detected in the xrs-6 and Scid cell lines that lack DNA-PK, this could be due to the existence of backup mechanisms for sensing DNA DSBs in mammalian cells. Alternatively, DNA-PK, rad3, and ATM might play more direct roles in DNA repair by recruiting the DNA repair machinery or controlling its activity. The convergence of DNA-PK, the TOR family, rad3, and ATM means that it will be important to decipher the relationships and distinctive features of these enzymatic systems. It will also be of great interest to determine whether there exist additional members of the DNA-PK$_{cs}$ subgroup of the PI3K superfamily and whether they are also employed in sensing or maintaining cellular integrity.

Experimental Procedures

Isolation of DNA-PK$_{cs}$ Clones

To obtain a homogeneous preparation of DNA-PK$_{cs}$, DNA-PK was purified as previously described (Lees-Miller et al., 1990) and then subjected to Mono Q–FPLC. DNA-PK$_{cs}$ (~350 μg) was cleaved at positions carboxy-terminal to methionine residues by treatment with cyanogen bromide. The resulting peptides were resolved by reverse-phase high pressure liquid chromatography, and protein microsequencing was performed using an Applied Biosystems 477A protein sequencer. Degenerate oligonucleotides corresponding to unambiguous peptide sequences were used to screen a HeLa λgt10 cDNA library at low stringency. The oligonucleotide (sequence 5′-AAGGGCGTGGCTGAG CAGAAGMGIGAGAAGGAG-3′) corresponding to peptide 18 (sequence KGVAEQKREK E) identified one positive clone. DNA sequencing was carried out by Sequenase Version 2.0 (United States Biochemical), and data was obtained at least twice on both strands. Sequence analyses were performed with the MacVector program package (International Biotechnologies) and by the program Pileup (GCG package; University of Wisconsin). Alignments were presented using the Boxshade program (K. Hofmann; Swiss Institute for Experimental Cancer Research).

Northern Blot Assays

Poly(A)$^+$ RNA was isolated from HeLa cells. After electrophoresis, 0.8% agarose gels were incubated in 0.5 M NaOH for 15 min to hydrolyze the RNA partially and then were neutralized in 10× SST, the RNA was transferred onto GeneScreen membranes (Dupont New England Nuclear), and hybridization and washing was accomplished according to the instructions of the manufacturer.

Purification of DNA-PK Components and Kinase Assays

DNA-PK was purified essentially as described previously (Blunt et al., 1995), and separation of DNA-PK$_{cs}$ from Ku was achieved by chromatography on phenyl–Sepharose (Pharmacia). DNA-PK peptide phosphorylation assays were conducted as previously described (Finnie et al., 1995) using a peptide derived from the amino-terminal transcriptional activation region of murine p53 (sequence EPPLSQEAFADLLKK). Sp1 overexpression, purification, and phosphorylation were as previously described (Jackson et al., 1990; Gottlieb and Jackson, 1993). Lipid kinase assays were conducted in 20 mM HEPES [pH 7.4], 10 mM $MgCl_2$ in the presence of 0.1 mM nonradioactive ATP and 10 μCi of [^{32}P]ATP (3000 Ci/mmol) at 30°C for 10 min. Lipids were dried under nitrogen, sonicated into the above buffer, and then presented (final concentration 20 μM) either alone or in combination with phosphatidyl serine (50 μM) and diacylglycerol (50 μM). Following addition of chloroform:methanol (1:1), lipids were extracted as described by Bligh and Dyer (1959) and separated by TLC on oxalate (1%)-sprayed plates using chloroform:methanol:ammonia:water (45:35:2:8). Spots were visualized using iodine and, after autoradiography, recovered by scraping and quantitated by liquid scintillation counting. Where appropriate, radiolabeled lipids were deacylated, and head groups were analyzed according to Stephens et al. (1991) and determined to be glycerophosphorylinositol (4)phosphate by cochromatography with an internal standard.

Immunological Procedures

Western blotting and detection was performed using the ECL system (Amersham). Immunoprecipitations were conducted essentially as described previously from crude nuclear extracts (Jackson et al., 1990). For in vivo [^{35}S]methionine labeling, HeLa cells were incubated in the presence of L-[^{35}S]methionine for 4 hr. To generate antisera against cloned portions of DNA-PK$_{cs}$, cDNA fragments were overexpressed in E. coli, electrophoresed on SDS–polyacrylamide gels, excised from the gel, and used to immunize rabbits at 4 week intervals.

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