Communication

Binding of Ku and c-Abl at the Kinase Homology Region of DNA-dependent Protein Kinase Catalytic Subunit*

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The DNA-dependent protein kinase (DNA-PK) controls the repair of double-stranded DNA breaks in mammalian cells. The protein kinase subunit of DNA-PK (DNA-PKcs) is targeted to DNA breaks by association with the Ku DNA-binding heterodimer. Here we show that a Ku association site is present at the carboxyl terminus of DNA-PKcs (amino acids 3002–3850) near the protein kinase domain. Correspondingly, the nuclear c-Abl tyrosine kinase that associates with DNA-PK also binds to the kinase homology domain. The c-Abl SH3 domain binds to amino acids 3414–3850 of DNA-PKcs. c-Abl phosphorlizes C-terminal fragments of DNA-PKcs, particularly amino acids 3414–3850. c-Abl phosphorylation of DNA-PKcs disassociates the DNA-PKcs-Ku complex. Thus, Ku and c-Abl provide opposing functions with regard to DNA-PK activity.

DNA double-strand break (DSB) repair occurs in eukaryotic cells following the formation of chromosome breaks by spontaneous damage or in V(D)J gene rearrangement of lymphoid cell differentiation. Strikingly, much of the same repair machinery is necessary for cell survival following ionizing radiation (IR) damage, known to include DSB repair (1, 2). In both the IR response and V(D)J recombination, genetic and molecular analysis has revealed that the components of the DNA-dependent protein kinase (DNA-PK) complex are essential in these repair responses.

DNA-PK consists of a complex of three proteins (reviewed in Ref. 3). DNA-PKcs is the 470-kDa catalytic subunit of DNA-PK containing a protein kinase homology domain at its C terminus. DNA-PKcs is structurally related to several other proteins that are implicated in radiation-induced checkpoint responses.

DNA-PK activity is positively regulated by binding of the 70- and 80-kDa Ku heterodimer (6–9). Ku binds to DNA in DSB repair reactions and in the absence of DNA-PKcs (10, 11). Potentially, the pools of Ku and DNA-PKcs are not always associated in eukaryotic cells, allowing activation and alternative regulation of the kinase by effector protein binding.

Although DNA-PK activity may elicit a DSB repair response to IR, DNA-PK substrates may have other functions. Recently, the nuclear tyrosine kinase, c-Abl, was shown to be activated by IR and to act upstream of c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) in signaling mechanisms that are initiated by DNA damage (12). c-Abl directly associates and is phosphorylated by DNA-PK following IR (13). Due to its growth-regulatory functions, c-Abl may regulate cell decisions of progression through the cell cycle or apoptosis, and c-Abl/DNA-PK interactions are responsive to DNA damage (13).

To better understand the significance of Ku and c-Abl associations with DNA-PK, we have examined the location of binding of these proteins. We find that Ku and c-Abl bind to the C terminus of DNA-PKcs near the kinase domain. Furthermore, c-Abl phosphorylates a DNA-PKcs fragment to which Ku binds and results in disassociation of DNA-PKcs from Ku.

EXPERIMENTAL PROCEDURES

In Vitro Transcription/Translation—A murine (22D6 pre-B cell) λ phage cDNA library was screened by reduced stringency hybridization procedures with two fragments of the human DNA-PKcs cDNA isolated by PCR, the 3'-untranslated region (14) and amino acids 1366–1996 (GenBank accession number U34994) (4). Phage cDNA inserts were excised by NcoI digestion and subcloned into pSGI+ (Stratagene).

Specific DNA-PKcs polypeptides were formed using a coupled in vitro transcription/translation methodology (Promega) with templates generated from the above cDNAs by PCR. 5' Primers contained a T7 RNA polymerase initiation sequence, ribosome binding site, and initiator methionine (GGAATCTTAAGGACTCACTATAGGGAGACCACCCATG) + 18–20 nucleotides specific to the 5' end of each DNA-PKcs fragment. 3' Primers were 21-mers. PCR products had the following amino acid coordinates and protein fragment sizes: DNA-PKcs-2 (515–527, 59.4 kDa), DNA-PKcs-1 (515–830, 36 kDa), DNA-PKcs-3 (770–1084, 35.7 kDa), DNA-PKcs-4 (1079–1533, 50.6 kDa), DNA-PKcs-14 (1520–1792, 30.9 kDa), DNA-PKcs-11 (1668–1796, 36 kDa), DNA-PKcs-5 (2005–2535, 51.1 kDa), DNA-PKcs-6 (2342–2574, 51 kDa), DNA-PKcs-15 (2726–2850, 56.8 kDa), DNA-PKcs-10 (3002–3450, 48.7 kDa), DNA-PKcs-13 (3002–3550, 59.5 kDa), DNA-PKcs-8 (3414–3850, 44.6 kDa), DNA-PKcs-15 (3414–4123, 80.8 kDa), and DNA-PKcs-9 (3757–4124, 41 kDa). 1–2 μg of PCR products purified by 0.7% agarose gel electrophoresis were used in the T7 in vitro transcription/translation.

Preparation of GST Fusion Proteins—Full-length c-Abl tyrosine kinase was engineered as a fusion protein with glutathione S-transferase (GST) from SF9 cells infected with the GST-c-Abl recombinant baculovirus (15, 16). SF9 lysates were prepared in 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 1 mM EDTA, 10% glycerol, and stored at –80 °C. GST-c-Abl was isolated by binding to glutathione-Sepharose 4B beads (Pharmacia Biotech Inc.) (0.5 ml of lysate/0.25 ml of beads) at 4 °C for 30 min. Beads were then washed four times in 10 mMTris·HCl (150 mM NaCl) and then eluted with freshly prepared 10 mM glutathione in 50 mM Tris-Cl, pH 9.5, 100 mM NaCl (final pH = 8.0) four times at 4 °C for 5 min each. Pooled fractions were passed through a 0.45-micron filter and assessed by a Bradford concentration assay and SDS-PAGE.

GST fusions to DNA-PKcs fragments 7, 8, and 9 were prepared by PCR incorporating a 5' BamHI site and a 3' XhoI site for subcloning into BamHI XhoI cleaved pGEX4T2, creating in-frame fusion proteins. GST protein (pGEX4T2) and GST-7, -8, and -9 proteins were produced by transformation of Escherichia coli BL21. Cultures of 1 liter in LB + 100 μg/ml ampicillin were grown to an OD600 of 0.6, adjusted to 1 ml isopropyl-

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1 The abbreviations used are: DSB, double-strand break; IR, ionizing radiation; DNA-PK, DNA-dependent protein kinase; DNA-PKcs, protein kinase subunit of DNA-PK; JNK, c-Jun terminal kinase; PCR, polymerase chain reaction; GST, glutathione S-transferase; PAGE, polyacrylamide gel electrophoresis.
1-thio-β-β-galactopyranoside, and grown for an additional 5 h at 25 °C. Cells were harvested, and 40-ml lysates were prepared in PBS + 1 mg/ml lysozyme, 1 μg/ml pepstatin, 10 mM EDTA for 15 min on ice, followed by freezing and thawing twice in liquid N₂. Lysates were adjusted to 1% Triton X-100, 150 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 μg/ml pepstatin, incubated on ice for 10 min, and sonicated with a Branson Sonifier 450. Sonicated lysates were spun in a Beckman J6 centrifuge with a JA-20 rotor at 13,000 rpm for 15 min. GST and GST fusion proteins were isolated by binding to 1–2 ml of GST beads (pre-equilibrated in PBS, 1% Triton X-100) by rocking for 30 min at 4 °C. Beads were then washed five times in PBS + 1% Triton X-100 prior to elution.

**Ku-DNA-PKcs and c-Ab-DNA-PKcs Binding—**Approximately 1 × 10⁶ human 293 cells were lysed in 1.0 ml of Nonidet P-40 lysis buffer (50 mM Tris, pH 8.0, 0.5% Nonidet P-40, 150 mM NaCl, 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin, 1 μg/ml aprotinin) on ice for 15 min. The NaCl concentration was then adjusted to 500 mM with 78 μl of 5 M NaCl so that Ku and DNA-PKcs dissociate (17). Lysates were also preclarified by adjusting to 6% polyethylene glycol 8000 for 10 min on ice and microcentrifugation for 15 min at 4 °C.

Aliquots of 293 lysates (0.5–1.0 mg) were mixed with equal amounts of each [³²P]DNA-PKcs in vitro translation product (10–20 μl), diluted to reduce the NaCl concentration to 150–170 mM (final volume = 350–450 μl), and incubated on ice for 1–3 h. Anti-Ku80 monoclonal antibody GE2-9.5 (18) was added for 1–2 h at 4 °C. Premixed protein A-Sepharose (10 μl) was added and incubated for an additional 30 min while rocking at 4 °C. Protein A-bound immunoprecipitates were washed four times with 1 ml of 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton X-100, and resuspended in 20 μl of 2 × 10 mM Tris-Cl, pH 7.0, 4% SDS, 720 mM 2-mercaptoethanol, 5 mg/ml bromphenol blue. Samples were boiled and fractionated on 10% SDS-PAGE as above. Anti-Ku70 monoclonal antibodies N3H10 (NeoMarkers) and AG4–7.5 were used similarly.

c-Ab binding to in vitro translated DNA-PKcs polypeptides was tested as above with 5 μg of GST-c-Ab, GST, or GST-c-Ab-SH3 in 20 μl of MB (10 mM Tris, pH 7.4, 150 mM NaCl, 1% Triton X-100, 10% glycerol, 0.1% leupeptin, and aprotinin) with equal amounts of in vitro translation product for 1–2 h at 4 °C. GST beads (10 μl) were added for 30 min with occasional resuspension and then washed four times in 1 ml of MB at 4 °C. Samples were prepared for 10% SDS-PAGE as above.

c-Ab Kinase Assays—GST-DNA-PKcs 7, 8, and 9 (2 μg) were mixed with GST-c-Ab (1 μg) and incubated at 30 °C in 1 × kinase buffer (25 mM Tris, pH 7.5, 75 mM KCl, 10 mM MgCl₂, 0.1 mM ATP and γ-³²P-ATP, 0.4 mM EDTA, 0.2 μM EGTA, 1 mM diithiothreitol). Samples were diluted by half in 2 × SB, boiled, and then fractionated on 10% SDS-PAGE followed by drying and autoradiography.

293 cells (5 × 10⁶) were lysed in 0.5 ml of Nonidet P-40 lysis buffer on ice for 30 min and centrifuged in a Beckman J6.1 rotor at 13,000 rpm for 15 min. Cell lysates were immunoprecipitated with anti-Ku80 antibody, GE2-9.5, and incubated on ice for 1–3 h. Anti-Ku80 monoclonal antibody GE2-9.5, absorbed to Protein A-Sepharose at 4 °C for 30 min, and washed four times with 1 ml of Nonidet P-40 lysis buffer and twice with 1 ml 1 × kinase buffer. Protein A beads for each sample were divided into two equal aliquots. Kinase reactions were conducted in 25 μl with or without addition of c-Ab (thrombin-cLEVD-cst-c-Ab) in 1 × c-Ab kinase buffer, γ-³²PATP, and ATP to 100 μM at 30 °C for 10 min. Reactions were microcentrifuged, the supernatant was quantitatively removed, and pellets were washed twice in 100 μl of Nonidet P-40 lysis buffer. Next supernatant and bead fractions were resuspended to equal volumes of 1 × SB, boiled, and fractionated on SDS-PAGE.

**Immunoprecipitation and Immunoblotting—**Immunoprecipitations were conducted with anti-Ku80 antibody, GE2-9.5, for 60 min on ice, followed by absorption onto Protein A-Sepharose. Protein A beads were washed twice in Nonidet P-40 lysis buffer, resuspended in 20 μl of 1 × SB, boiled for 5 min, and fractionated on SDS-PAGE. Proteins were transferred to polyvinylpyrrolidone difluoride membranes (NEN Life Science Products) by using a Bio-Rad semi-dry electrophoretic transfer apparatus at 20 V for 45 min in 48 mM Tris, 39 mM glycine, 1.3 mM SDS, 20% methanol, pH 9.2. Blots were blocked in PBS + 3% bovine serum albumin overnight. Primary antibodies, polyclonal anti-DNA-PKcs antibodies (15), or anti-Ku70 (M19, Santa Cruz Biotechnology) were used at 1–2 μg/ml in PBS for 60 min. Blots were then washed five times in PBS + 0.2% Tween 20, incubated with Protein A/G-horseradish peroxidase (Pierce) for 30–40 min in PBS + 1% bovine serum albumin, and extensively washed in PBS + 0.2% Tween 20. Blots were developed using a Chemiluminescence Renaissance Kit (NEN Life Science Products).

**RESULTS AND DISCUSSION**

**Ku Association with DNA-PKcs—**To determine the epitopes governing Ku association with DNA-PKcs, fragments of the DNA-PKcs polypeptide were prepared from mouse DNA-PKcs cDNAs. Fourteen different DNA-PKcs fragments, representing the entire open reading frame (Fig. 1), were synthesized by in vitro transcription/translation. Each in vitro translation reaction produced a polypeptide of the expected size by SDS-PAGE (data not shown).

As a source of Ku, 293 cell lysates were prepared by high salt extraction that disassociates Ku from DNA-PKcs, but does not disrupt the Ku heterodimer (9, 17, 18). Following co-incubation of in vitro translation products with lysates at 150 mM NaCl, immunoprecipitations were formed with the anti-Ku antibody, GE2-9.5, absorbed to Protein A-Sepharose, and then extensively washed. In parallel, the in vitro translation products were incubated with 293 lysates and Protein A-Sepharose alone was compared. We found that DNA-PKcs-8 and DNA-PKcs-10 (amino acids 3414–3850 and 3002–3450, respectively) yielded significantly greater co-immunoprecipitated translation products than each of the other DNA-PK fragments or the Protein A only controls (Fig. 2). Also, DNA-PKcs-8 and DNA-PKcs-10 translation products were not immunoprecipitated with GE2-9.5 without addition of the 293 cell lysate (data not shown). DNA-PKcs-9 showed increased binding, but equal binding to the Protein A negative control, arguing that this association is not specific to Ku. A specific association between Ku and DNA-PKcs-8 and -10 was also observed with two anti-Ku70 monoclonal antibodies, N3H10 and AG4-7.5 (data not shown). Thus, Ku binds to DNA-PKcs through an 848-amino acid region at least partially overlapping the protein kinase homology domain.

The Ku binding region is N-terminal to conserved residues that are likely to be involved in phosphotransfer catalysis for DNA-PK (motifs DXXXX, amino acids 3917–3922, and DFG, amino acids 3936–3938, respectively (4, 19)). Therefore, Ku association might contribute to structural changes activating the nearby kinase core domain or make it more available to protein substrates.

c-Ab Binding to DNA-PK—We tested the localization of c-Ab binding to DNA-PKcs in a similar manner as for Ku. Equal quantities of DNA-PKcs in vitro translation products were incubated with 5 μg of GST-c-Ab or GST proteins (Fig. 3B), absorbed onto glutathione-Sepharose beads, and washed. We observed specific binding of c-Ab to DNA-PKcs fragments 8, 9, and 10 (Fig. 3A). Likewise, DNA-PKcs-15, spanning fragments 8–10 and including the entire kinase homology domain, also associated with c-Ab. GST protein and beads gave relatively weak binding to these and other fragments. DNA-PKcs-11 bound to GST-c-Ab and GST equally indicating an association that was not specific to c-Ab. Thus, c-Ab binds to DNA-PKcs by recognizing an epitope that could be involved in phosphotransfer catalysis.
the entire carboxyl terminus of DNA-PKcs (3002–4124) including the kinase homology domain.

The carboxyl-terminal DNA-PKcs fragments 8, 9, and 10 were also examined for binding to the c-Abl SH3 domain, as we previously showed that c-Abl and DNA-PKcs in part associate via c-Abl SH3 (13). We found that GST-c-Abl-SH3 strongly binds to DNA-PK fragment 8, but not appreciably with either fragments 9 or 10 (Fig. 3C). Therefore, the association between c-Abl and DNA-PKcs may occur by multiple binding sites: c-Abl-SH3 with 3414–3850 and additional c-Abl epitopes with 3002–3450 and 3757–4124.

c-Abl Phosphorylation Disassociates the DNA-PK Complex—c-Abl phosphorylation of C-terminal DNA-PK fragments was also studied in vitro. GST fusion proteins of DNA-PK-7, -8, and -9 were isolated. DNA-PKcs fragment 8, which binds to c-Abl and Ku, was most actively phosphorylated in vitro by c-Abl (GST-DNAPK-8, Fig. 4A). DNA-PK-9 is not appreciably phosphorylated by c-Abl, and DNA-PK-7, which is not stably associated with c-Abl in vitro compared with DNA-PKcs-8, has lower phosphorylation levels. DNA-PK-8 phosphorylation is significantly lower than GST-CRK (amino acids 120–225), anther substrate that stably binds to c-Abl (20) (data not shown).

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DNA-PK-8 as effectively as c-Abl (Fig. 4A). Thus, although c-Abl may utilize the SH3 domain as part of its interaction with the kinase domain of DNA-PKcs, there is no requirement for this domain in phosphorylating DNA-PK polypeptide substrates. c-Abl kinase could inhibit DNA-PK by phosphorylation that may or may not induce disassociation of the DNA-PK complex. To examine this issue, we isolated DNA-PK by anti-Ku80 immunoprecipitation from 293 cell lysates. Following washing and resuspension in kinase buffer, active c-Abl was added with \([\gamma-32P]ATP\). To ensure that phosphorylation was not due to DNA-PK, wortmannin was added to inhibit DNA-PK (4). Beads were then centrifuged, and the supernatant and bead fractions were separated by 5% SDS-PAGE. We observed that all of the phosphorylated DNA-PKcs was released from Ku in the immunoprecipitation complexes (Fig. 4B). In the absence of c-Abl, wortmannin completely inhibited DNA-PK autophosphorylation (Fig. 4B). Similarly, Ku and DNA-PKcs are retained within the Ku immunoprecipitates incubated in kinase buffer without c-Abl as evidenced by immunoblotting with anti-DNA-PK and anti-Ku antibodies (Fig. 4C). Therefore, c-Abl activity disassociates DNA-PK into separate Ku and DNA-PKcs components. Unlike Ku, c-Abl appears to negatively regulate DNA-PK (13). Down-modulation of DNA-PK activity by c-Abl and possibly other proteins may occur during normal cell growth. Alternatively, c-Abl interactions may play an important role in radiation responses by truncating DNA damage signaling to the time immediately following the recognition of chromosome breaks. In such a capacity, DNA-PK complexes would have to reform at new DNA damage sites to reinitiate a signaling step. Thus, DNA repair functions of DNA-PK may not require the full activity of both DNA-PKcs and Ku components all of the time.

DNA-PK autophosphorylation also inactivates DNA-PK in a mechanism where each component of the heterotrimeric complex is phosphorylated and Ku disassociates from DNA-PKcs (21). Autophosphorylation of the catalytic subunit rather than Ku specifically inactivates DNA-PK, since only unphosphorylated DNA-PKcs can restore DNA-PK activity in addback experiments (21). Possibly the sites of autophosphorylation and c-Abl phosphorylation are in the same carboxyl-terminal region of DNA-PKcs, as both mechanisms appear to affect Ku association in a similar way.

Atm, the ataxia telangiectasia gene product, is a protein kinase related to DNA-PK (22). Ataxia telangiectasia cells are also IR-sensitive and show chromosome instability defects (23). No activating proteins or additional subunits of Atm are known. Interestingly, Atm-c-Abl complexes have been found, and the kinase homology region of ATM is sufficient to activate c-Abl (24, 25). Thus, it is likely that DNA-PKcs and Atm use similar epitopes for associations with the c-Abl protein. The position of Ku-DNA-PKcs binding also roughly corresponds to that of rapamycin-binding protein, FKBP12, to FRAP, another member of the PIK family (5, 26, 27). FKBP12-rapamycin binding inhibits FRAP function on downstream targets such as p70 S6 kinase and cyclin-dependent kinases (28). Thus, the N-terminal regions flanking kinase homology domains of PIK family members may well be used as a general strategy for modulating kinase activity in vivo.

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