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Isolation of Ku70-binding proteins (KUBs)

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ABSTRACT

DNA-dependent protein kinase (DNA-PK) plays a critical role in resealing DNA double-stand breaks by non-homologous end joining. Aside from DNA-PK, XRCC4 and DNA ligase IV, other proteins which play a role(s) in this repair pathway remain unknown; DNA-PK contains a catalytic subunit (DNA-PKcs) and a DNA binding subunit (Ku70 and Ku80). We isolated Ku70-binding proteins (KUB1–KUB4) using yeast two-hybrid analyses. Sequence analyses revealed KUB1 to be apolipoprotein J (apoJ), also known as X-ray-inducible transcript 8 (XIP8), testosterone-repressed prostate message-2 (TRPM-2) and clusterin. KUB2 is Ku80. KUB3 and KUB4 are unknown, >10 kb transcripts. Interactions of apoJ/XIP8 or KUB3 with Ku70 were confirmed by co-immunoprecipitation analyses in MCF-7:WS8 breast cancer or IMR-90 normal lung fibroblast cells, respectively. The interaction of apoJ/XIP8 with Ku70 was confirmed by far-western analyses. Stable over-expression of full-length apoJ/XIP8 in MCF-7:WS8 caused decreased Ku70/Ku80 DNA end binding that was restored by apoJ/XIP8 monoclonal antibodies. The role of apoJ/XIP8 in ionizing radiation resistance/sensitivity is under investigation.

INTRODUCTION

Ionizing radiation (IR) causes a spectrum of DNA lesions (reviewed in 1–5), including DNA single-strand breaks (SSBs), double-strand breaks (DSBs), DNA–protein crosslinks (intra- and inter-strand) and apurinic/apyrimidinic sites. Unrepaired DSBs cause loss of survival (6), G_2/M cell cycle checkpoint arrest (7) and chromosome aberrations (8). The consequences of DNA damage tolerance (i.e. unrepaired or misrepaired DNA lesions which go undetected) after any genetic insult may lead to increased rates of mutation and ultimately carcinogenesis (8), as found in patients prone to hereditary non-polyposis colorectal cancer (HNPCC) that carry one mutated allele for DNA mismatch repair genes (9–11).

To repair, recover and survive following IR, mammalian cells must first recognize DNA lesions. A few pathways for the recognition and repair of DSBs have been elucidated. Two types of DSB repair occur within the first few hours after IR treatment (8,12): homologous and non-homologous recombination. At least two processes for non-homologous end joining (NHEJ) of DSBs are thought to occur in mammalian cells, one involving the DNA-dependent protein kinase (DNA-PK) complex (13), the other involving the RAD50/MRE-11/p95 proteins (14); it is possible that these processes represent one pathway, since they are epistatic in yeast. DNA-PK-mediated NHEJ is thought to be the major pathway in mammalian cells for the repair of DSBs produced after IR or other DNA damaging agents, but also plays a role in V(D)J recombination during T and B cell development (reviewed in 13).

To date, the known components of the DNA-PK complex are the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}, 460 kDa) and the Ku autoantigen. The Ku autoantigen was initially discovered in several autoimmune diseases (15,16) and is composed of a nuclear heterodimer of 70 kDa (Ku70) and 80 kDa (Ku80) proteins (reviewed in 17–20). Ku70/Ku80 was also identified as human DNA helicase II (HDH II), a novel ATP-dependent DNA unwinding enzyme (21).

DNA-PK is a nuclear serine/threonine kinase which requires DNA ends for catalytic activity (reviewed in 17–20). DNA-PK is selectively activated by binding to the ends of linear double-strand DNA (22). The actual mechanism by which DNA-PK participates in the physical resealing of a DSB is poorly understood. It is possible that the primary function of the DNA-PK complex is to act as a DNA damage sensor, which simultaneously recruits other components of the DNA repair machinery to DNA lesions and also transduces signals which affect cell cycle regulation and apoptosis (7). Additional participants in the DNA-PK-mediated NHEJ repair pathway appear to include X-ray cross-complementing protein 4 (XRCC4) (23), DNA ligase IV (23) and, possibly, poly(ADP-ribose) polymerase (PARP) (24). There are likely to be other proteins involved in this NHEJ process of DSB repair. These may include DNA ligases, nucleases, helicases, single-strand-binding proteins and polymerases for DNA end processing prior to accurate DNA strand rejoining.

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In addition, numerous regulatory, modifying and cofactor proteins are probably involved. The apparent absence of known functional proteins in DNA-PK-mediated NHEJ repair prompted us to search for Ku-binding proteins (KUBs). We describe the cloning of four such cDNAs/proteins by yeast two-hybrid analyses using Ku70 as 'bait'.

MATERIALS AND METHODS

Yeast two-hybrid and DNA sequence analyses

The yeast two-hybrid system, detailed protocols [MatchMaker Two-Hybrid System 2 Protocol, Clontech Laboratories (PT1030-1); 25], various yeast strains and plasmids were generous gifts from Dr S. Elledge (Baylor College of Medicine, Houston, TX). Briefly, full-length human Ku70 cDNA (from Dr W. Reeves, University of North Carolina, Chapel Hill, NC) was subcloned in-frame into the pAS2 vector to form a hybrid protein with the GAL4 DNA binding domain (GAL4-DBD) at its N-terminus. The fused GAL4–Ku70 cDNA was sequenced and transformed into Y190 yeast, which carried both GAL4-responsive LacZ (for detection) and GAL4-responsive HIS3 (a selection marker) genes. After confirming GAL4–Ku70 expression and lack of self-activation in Y190 yeast, a yeast two-hybrid screen using a 'prey' human liver cDNA library [cDNAs were fused with the GAL4 activation domain (GAL4-AD), catalogue no. HL4024AH; Clontech, Palo Alto, CA] was performed to screen for KUBs. cDNA plasmids from the liver library were isolated from each positive yeast clone (i.e. His^+, β-gal^+ clones) and the interaction was reconfirmed by co-transfection into Y190 yeast with pAS2-GAL4-Ku70 plasmid DNA as described above. None of the isolated clones self-activated. Yeast expressing p53 or lamin B were obtained from Dr Elledge and were used for specificity tests to eliminate false positive clones (26,27). Sequences of each isolated cDNA clone were screened for homology to known genes using the GenBank database.

Tissue culture conditions and IR treatments

MCF-7:WS8 human breast cancer cells, a clonal derivative of MCF-7, were obtained from Dr V. Craig Jordan (Northwestern University, Chicago, IL). MCF-7:WS8 cells were dependent on estrogen for maximal growth and estrogen receptor-mediated gene expression (28). Hereafter, MCF-7:WS8 cells will be referred to as MCF-7 cells. MCF-7 cells were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) as described (29). Growth medium was changed daily, since KUB1 (i.e. apoJ/XIP8; Table 1) protein expression increased when cells reached confluency or were starved for growth factors (i.e. serum-depleted); this gene is repressed by testosterone (30). Human HepG2 hepatocellular carcinoma, HeLa and IMR-90 normal human lung fibroblast cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% FBS at 37°C in a humidified tissue culture incubator with a 10% CO_2/90% air atmosphere. For IR treatments, log phase MCF-7 cells were irradiated using a ^{137}Cs source at a dose rate of 5.79 Gy/min. Control cells were mock-irradiated under identical conditions. All cells were free from mycoplasma infection and monitored monthly.

Overexpression of full-length KUB1 (i.e. apoJ/XIP8) cDNA in MCF-7 cells

Full-length human apoJ/XIP8 cDNA, obtained from Dr M. Tenniswood (Department of Biological Sciences, University of Notre Dame, Notre Dame), was subcloned into the pTarget (Promega, Madison, WI) mammalian expression vector driven by the CMV-promoter. MCF-7 cells were transfected with either pTarget full-length apoJ/XIP8 or the pTarget empty vector using Lipofectamine (Gibco BRL, Gaithesburg, MD). After growth in RPMI medium with 200 μg/ml G418 for 14 days, colonies of stable transfectants were isolated and tested for apoJ/XIP8 protein expression by western immunoblot analyses as described below.

Overexpression of apoJ/XIP8 and Ku70 proteins in bacteria

The pET bacterial expression system (Novagen, Madison, WI) was used to produce apoJ/XIP8, β-galactosidase (β-gal), full-length Ku70 and C-terminal deleted Ku70 (Ku70 *Stu*I del) proteins. cDNAs were constructed in the pET28a vector, then transformed into B834(DE3) bacteria. Transformed bacteria were grown in NZCYM medium (Difco Laboratories, Detroit, MI) and protein expression was induced by 1.0 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were then harvested and lysed in 2× SDS–PAGE loading buffer (31) for far-western analyses (below). All plasmid constructs were confirmed by DNA sequencing.

Antibodies

A mouse monoclonal antibody (clone 41D) raised against the secretory form of clusterin (apoJ/XIP8) was purchased from Upstate Biotechnology (Lake Placid, NY). This antibody detected both precursor (60 kDa) and secretory forms of apoJ/XIP8 (32). Five mouse monoclonal apoJ antibodies (Abs 1, 5, 6, 10 and 11) and one rabbit polyclonal apoJ antibody (RαJβ) were obtained from Dr J. Harmony (Department of Biochemistry, University of Cincinnati, Cincinnati, OH) (33). Mouse monoclonal antibodies raised against human Ku70 (N3H10) and Ku80 (N9C1) were obtained from Dr R. Burgess (University of Wisconsin–Madison, Madison, WI) (34). A mouse monoclonal antibody which specifically recognized the Ku70/Ku80 heterodimer (Ab 162) was obtained from Dr W. Reeves (University of North Carolina, Chapel Hill, NC) (35). Rabbit polyclonal Ku70 antibodies (Abs 2, 5, 6, 7, 9, 10 and 11) were obtained from Dr B. Prabhakar (Departments of Microbiology and Immunology, University of Illinois–Chicago, Chicago, IL) (36). A goat polyclonal Ku70 antibody (C19), horseradish peroxidase (HRP)-conjugated secondary antibodies and control IgGs for co-immunoprecipatation were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Generation of polyclonal antiserum against a truncated KUB3 polypeptide

Using the partial KUB3 cDNA insert (1.2 kb) originally isolated from the human liver cDNA library by yeast two-hybrid analysis (Table 1), a λgt10 human liver cDNA library made from the same RNA source (Clontech) was screened. A larger insert corresponding to KUB3 (i.e. 1.4 kb, which contained the sequence of the original fragment) was isolated, sequenced and used for both northern blot hybridization and the generation of rabbit polyclonal antiserum. The partial KUB3 cDNA insert was subcloned into the pET-28c(+) vector for protein expression/purification and the open reading frame (ORF) (corresponding to codons in-frame

with the GAL4-AD) was confirmed by DNA sequencing. BL21(DE3) bacteria were transformed and His-truncated KUB3 fusion protein was induced by 1.0 mM IPTG, then purified by nickel resin affinity column chromatography and concentrated as per the manufacturer's instructions (Novagen). Polyclonal antiserum (referred to as KL-1 antibody) against the KUB3 polypeptide, as well as preimmune sera, were then generated in rabbits. The KL-1 polyclonal antiserum detected a predicted 36 kDa polypeptide in extracts from bacteria expressing truncated KUB3–His protein, but not from non-transformed bacteria. Likewise, KL-1 detected a band of ~50 kDa (as predicted from GAL4-AD + the N-terminally truncated KUB3 insert) in yeast, but not in yeast containing other cDNA plasmids. Preimmune sera from the same animal did not detect any bands in whole cell extracts from human, yeast or bacterial cells at similar 1:500 dilutions. Two polypeptides with estimated molecular weights of ~300 and ~220 kDa were detected in all human cells examined.

Preparation of whole cell lysates and western immunoblot analyses

For whole cell lysate preparations, cells were lysed in RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS). Supernatants were collected by centrifugation (16 000 *g*, 5 min) at 4°C and protein concentrations were determined by Bradford (Bio-Rad, Richmond, CA) assays. Protein (40–50 µg) from whole cell lysates were separated by 8% SDS–PAGE under reducing (1.0% β-mercaptoethanol) conditions and transferred to Immobilon-P membranes (Millipore Corp., Bedford, MA). Steady-state apoJ/XIP8 protein levels were detected using horseradish peroxidase (HRP)-conjugated anti-mouse secondary antibodies and SuperSignal chemiluminescence (Pierce, Rockford, IL). Equal loading was confirmed by Ponceau S dye staining or by monitoring Ku70 protein levels. All experiments (western, far-western and northern blots, co-immunoprecipitation and gel shift assays) presented in this paper were performed at least three times.

Far-western and southwestern blot analyses

Bacteria expressing Ku70, Ku70 *Stu*I del, β-gal and apoJ/XIP8 were lysed and boiled in SDS–PAGE loading buffer (31). Proteins were then separated by SDS–PAGE and transferred to Immobilon-P membranes. After further denaturation in HEPES buffer with 6 M guanidine hydrochloride, proteins were renatured as described (37). Blots were incubated with either [^{35}S]methionine-labeled apoJ/XIP8 or Ku70 protein (far-western) or [^{32}P]DNA probe (as described below, southwestern) and monitored by PhosphorImager (Molecular Dynamics, Sunnyvale, CA) analyses. The TNT system (*in vitro* transcription and translation system; Promega, Madison, WI) was used to label proteins with [^{35}S]methionine as per the manufacturer's directions.

Co-immunoprecipitation of apoJ/XIP8, KUB3 and Ku70 proteins

Whole cell lysates prepared from MCF-7 cells overexpressing apoJ/XIP8 (MCF-7-apoJ/XIP8) were separately incubated with various antibodies (as indicated) in HEPES buffer (25 mM HEPES, pH 7.9, 25 mM NaCl, 5 mM $MgCl_2$) for 1 h at 4°C (cold room) with constant rocking. Five mouse monoclonal apoJ antibodies (Abs 1, 5, 6, 10 and 11) and one rabbit polyclonal apoJ antibody (RαJβ) were separately used to co-immunoprecipitate Ku70. Seven rabbit polyclonal Ku70 antibodies (Abs 2, 5, 6, 7, 9, 10 and 11) and one goat polyclonal Ku70 antibody (C19) were separately used to co-immunoprecipitate apoJ. Protein A/G–agarose beads (Santa Cruz Biotechnology) were then added and binding reactions performed overnight at 4°C (cold room) with constant rocking. Precipitated proteins were collected by centrifugation (550 *g*, 2 min) at 4°C and washed three times with HEPES buffer. Bound proteins were eluted by boiling for 5 min in SDS–PAGE loading buffer (31). After centrifugation (16 000 *g*, 5 min) at 4°C, supernatant proteins were separated by SDS–PAGE and detected by western immunoblot analyses. Ku70 was detected using the anti-Ku70 antibody (N3H10) and apoJ/XIP8 (60 kDa) was detected using the anti-clusterin (41D) antibody.

Since HeLa cells constitutively express apoJ/XIP8, they were used to demonstrate a normal physiological association between this protein and Ku70. Log phase HeLa cells were extracted (2% Triton X-100, 150 mM NaCl, 2.5 mM KCl, 5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 10 mM EDTA, 25 mM Tris–HCl, pH 7.4, 1.0 mM dithiothreitol, 10% glycerol and 1.0 mM phenylmethylsulfonyl fluoride) and clarified by centrifugation (10 000 *g*, 10 min, 4°C). Whole cell extracts (100 µg) were then incubated with anti-Ku70 antibody (N3H10) or mouse IgG for 3 h at 4°C with constant rotation of reactants. Protein G–Sepharose (Boehringer Mannheim, Indianapolis, IN) was added and the mixture was incubated for 1 h at 4°C. Co-immunoprecipitates were collected by centrifugation and Ku70 or apoJ/XIP8 protein levels were monitored by western immunoblotting.

Western blot analyses indicated that KUB3 expression was elevated and constitutive in IMR-90 cells compared to MCF-7, HepG2 or U1-Mel cells. For KUB3–Ku70 co-immunoprecipitation analyses, 100 µg of IMR-90 whole cell extract were incubated with 10 µg of rabbit polyclonal anti-human Ku70 antibody 6 or goat polyclonal anti-human Ku70 (C19) antibody overnight at 4°C. Protein G–agarose (Pierce) was added and the mixture was incubated for 2 h at 4°C. Co-immunoprecipitates were collected by centrifugation and separated by 5% SDS–PAGE (for KUB3) and 10% SDS–PAGE (for Ku70), then detected using the Ku70 (C19) antibody or the rabbit polyclonal anti-human KUB3 antiserum (KL-1).

Northern blot analyses

Total RNA was extracted from log phase human HepG2, MCF-7 or U1-Mel cells using RNAzol (Tell-Test, Friendswood, TX). RNA samples (30 µg) were analyzed by northern blot analyses using ^{32}P-labeled, random primed partial KUB3 or KUB4 cDNA inserts. Equal loading of northern blots was accomplished by ethidium bromide-stained gels and by 36B4 transcript levels as described (38).

Ku70/Ku80 DNA end binding assays

A gel electromobility shift assay was used for the determination of Ku70/Ku80 DNA end binding activity as previously described (22). Briefly, a DNA probe was made by annealing two complimentary, synthesized oligonucleotides containing randomly chosen sequences. Sequences used were 5′-GATCGATTAGTTAC-GTAACGTTATGA-3′ and 5′-GATCTCATAACGTTACGTAAC-TAATC-3′ (22), purified by 12% PAGE (39). The DNA probe was labeled using [α-^{32}P]dCTP and the Klenow fragment of DNA polymerase I (New England Biolabs, Beverly, MA) as per the

manufacturer's directions. Unincorporated nucleotides were removed by Sephadex G-25 spin column chromatography (Boehringer Mannheim). Radiolabeled DNA probe (1 ng, 10 000–15 000 c.p.m.) was then incubated with whole cell lysates (1.0 µg) in binding buffer (100 mM Tris–HCl, pH 7.5, 10% glycerol, 0.5 mM DTT) for 20 min at room temperature. Samples were then electrophoresed in a non-denaturing 5% polyacrylamide gel using 0.25× TBE buffer (31) at room temperature for 2–3 h at 150 V. Gels were dried on Whatman 3M paper and exposed to X-ray film at –80°C for 3 h. For gel supershift experiments, antibodies were added to the binding reaction after the first 10 min incubation and incubated for another 10 min prior to PAGE. DNA binding activity was quantitated by PhosphoImager (Molecular Dynamics).

RESULTS

Isolation of KUBs by yeast two-hybrid analyses

A yeast two-hybrid screen using human Ku70 as 'bait' and a human liver cDNA library as 'prey' was performed as described in Materials and Methods (Table 1). Several positive clones were isolated (i.e. 518), of which we rescued the plasmid DNA of 108 randomly selected clones. Eighty-three plasmid DNAs were isolated and 24 of these analyzed, based on differences in insert sizes, by DNA sequencing. Sequence analyses indicated that 19 of the 24 clones were identical to human apoJ (40), also known as clusterin (41), testosterone-repressed prostate message-2 (TRPM-2) (41) and X-ray-inducible transcript 8 (XIP8) (38) (designated KUB1; Table 1). The binding regions between Ku70 and apoJ/XIP8 have been explored and will be reported elsewhere (C.-R.Yang, E.Odegaard, G.Zhu, B.Aronow, J.K.Harmony and D.A.Boothman, submitted for publication). Two of 24 clones were identical to Ku80 (designated KUB2; Table 1), which is a known protein that tightly associates with Ku70 (17). KUB3 (two of 24) and KUB4 (one of 24) were also isolated, but these clones were unknown with respect to registered GenBank sequences (Table 1). No apparent sequence overlap between KUB3 and KUB4 cDNAs were noted. Due to their large transcript sizes (>10 kb; below) we cannot be sure that these genes do not represent cDNAs made from the same transcript.

Limited specificity testing using yeast two-hybrid analyses were then performed (Table 1). apoJ/XIP8 interacted with full-length Ku70, but not with the Ku70 *Stu*I del mutant, p53 or lamin B. The Ku70 *Stu*I del mutation eliminated 92 amino acids (276 bp fragment) near the Ku70 C-terminus, which is important for Ku80-independent DNA end binding activity (Fig. 2B; 42,43). These data suggested that apoJ/XIP8 may interact with Ku70 at its C-terminus to modify Ku80-independent DNA end binding. In contrast, KUB2 (i.e. Ku80), KUB3 and KUB4 interacted with full-length Ku70 and with Ku70 *Stu*I del mutant, but not with p53 or lamin B (Table 1).

Partial analyses of KUBs3 and 4

KUB3 and KUB4 were unknown genes based on GenBank searches. We analyzed their expression patterns in various human cancer cells (i.e. HepG2, U1-Mel and MCF-7; Fig. 1A). Single transcript bands for KUB3 or KUB4 were detected in all cells examined by northern blot analyses, with sizes >10 kb.

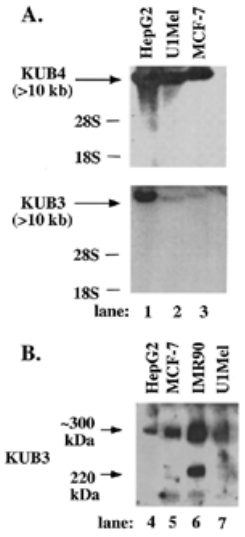

Figure 1. Partial analyses of KUB3 and KUB4 by northern and western blot analyses. Log phase HepG2, U1-Mel, MCF-7 and IMR-90 cells were extracted for total RNA and whole cell protein lysates as described in Materials and Methods. (**A**) Northern blots of total RNA (30 µg) were probed with ^{32}P-labeled KUB4 or KUB3 cDNAs. KUB3 and KUB4 transcripts were estimated to be >10 kb and were observed as single bands. All cell lines tested expressed KUB3 and KUB4 mRNA, although levels of KUB3 were much lower in MCF-7 and U1-Mel than in HepG2 cells (lanes 1–3). rRNA (18S and 28S) positions are indicated and equivalent loading was confirmed using ethidium bromide staining. (**B**) Equal amounts (40 µg) of whole cell protein extract were separated by 5% SDS–PAGE. Equal loading was monitored using Ponceau S staining. Proteins were transferred to immobilon-P membranes and probed with the KL-1 polyclonal antiserum, which was made against a truncated KUB3 polypeptide as described in Materials and Methods. Two KUB3-related polypeptides were detected at ~300 and ~220 kDa.

Table 1. Cloning of KUBs by yeast two-hybrid analyses[a]

Isolated clones[b]	Identity[c]	Ku70 interactions		Specificity tests (His$^+$, β-gal$^+$)		
		(His$^+$)	(β-gal$^+$)	Ku70 *Stu*I del[d]	p53	Lamin B
KUB1	apoJ/XIP8	+	+	–	–	–
KUB2	Ku80	+	+	+	–	–
KUB3	Unknown	+	+	+	–	–
KUB4	Unknown	+	+	+	–	–

[a]Yeast two-hybrid analyses using human full-length Ku70 cDNA as 'bait' and a human liver cDNA library as 'prey' was performed as described in detail in Materials and Methods. Ku70 interactions and specificity tests were performed as described in Materials and Methods. A positive (+) reaction was defined by growth in histidine-deficient medium and the production of β-galactosidase (β-gal).

[b]Positive yeast colonies were isolated and analyzed as described in Materials and Methods and Results. A number of clones (24 of 83) were selected for DNA sequence analyses based on various lengths of their inserts. Nineteen of 24 clones were KUB1 (i.e. apoJ/XIP8; see below), two of 24 were KUB2 (i.e. Ku80), two of 24 were KUB3 and one of 24 was KUB4.

[c]KUB1 cDNA was 100% identical to apolipoprotein J (apoJ; 40), also known as X-ray-inducible transcript-8 (XIP8; 38) or testosterone-repressed prostate message-2 (TRPM-2; 58). KUB2 was 100% identical to Ku80. The partial nucleotide sequences for KUB3 and KUB4 genes have been deposited in the GenBank database under GenBank accession nos AF078164 and AF078528, respectively. These two cDNAs, with estimated transcript sizes >10 kb, contained no identity with any known genes in GenBank.

[d]Ku70 *Stu*I del mutation eliminated 92 amino acids (276 bp fragment) near the Ku70 C-terminus.

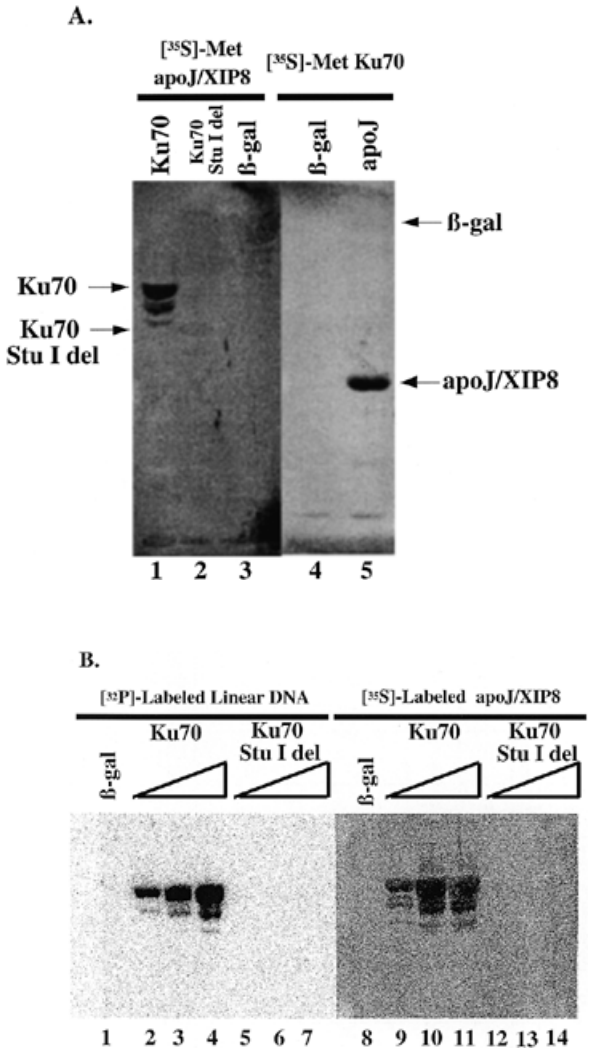

Figure 2. Far-western blot analyses of Ku70–apoJ/XIP8 interactions. (**A**) Lysates were prepared from bacteria overexpressing human Ku70, Ku70 *Stu*I del or β-gal proteins and transferred to Immobilon-P membranes after SDS–PAGE separation. Proteins were renatured on the membrane, then probed with *in vitro* translated [^{35}S]methionine-labeled apoJ/XIP8 or [^{35}S]methionine-labeled Ku70. Equal loading was monitored using Ponceau S staining. Only Ku70 and apoJ/XIP8 interacted. apoJ/XIP8 protein bound only Ku70 and not to the Ku70 *Stu*I del or the β-gal proteins. (**B**) Southwestern analyses were used to examine Ku70 DNA binding activity (Ku80-independent). Increasing amounts of Ku70 or Ku70 *Stu*I del proteins were loaded, renatured and probed with radiolabeled linear DNA or apoJ/XIP8. Only Ku70 bound linear DNA or apoJ/XIP8. Far-western and southwestern blots were analyzed as described in Materials and Methods. β-Gal was included as a negative control.

KUB4 mRNA was relatively abundant in all cells, while KUB3 was expressed at high levels in HepG2 cells and very low levels were found in U1-Mel and MCF-7 cells (Fig. 1A). The apparent differential expression of these gene transcripts may represent differences in steady-state levels of KUB3 compared to KUB4 in various cell lines and suggests that the two cDNAs are copies of two different transcripts.

Within the KUB3 cDNA we were able to find a continuous ORF, which was in-frame with the GAL4-AD in the yeast two-hybrid plasmid cDNA construct. Using this ORF we generated a rabbit polyclonal antiserum (i.e. KL-1), which detected KUB3 polypeptides of ~300 and ~220 kDa (Fig. 1B). Similar steady-state KUB3 protein levels were found in HepG2, MCF-7 and U1-Mel cells. Much higher protein levels were detected in IMR-90 cells. The relationship between the 300 and 220 kDa proteins was not determined and must await the isolation of full-length KUB3 cDNA (estimated to be >10 kb). A very short ORF was found for KUB4, however, we have not been able to generate a rabbit polyclonal antiserum from a protein derived from this limited reading frame. No unique motifs in KUB3 or KUB4 were found in the limited DNA sequences of these two genes. Due to the immense sizes of the KUB3 and KUB4 unknown transcripts, we concentrated on the apoJ/XIP8 protein.

Interaction of Ku70 and apoJ/XIP8 proteins by far-western blot analyses

Far-western blot analyses were initially used to confirm the Ku70-apoJ/XIP8 protein–protein interaction. Lysates from bacteria expressing Ku70, the Ku70 *Stu*I del mutant, β-gal and apoJ/XIP8 were prepared as described in Materials and Methods. Separated and renatured membrane-bound proteins were incubated with either [^{35}S]methionine-labeled apoJ/XIP8 or Ku70 proteins. [^{35}S]methionine-labeled apoJ/XIP8 interacted with Ku70, but not with β-gal or Ku70 *Stu*I del proteins (Fig. 2A, lanes 1–3, and B, lanes 8–14). Similarly, [^{35}S]methionine-labeled Ku70 protein bound only apoJ/XIP8 and not to β-gal (Fig. 2A, lanes 4 and 5). Southwestern blot analyses showed that only full-length Ku70, but not Ku70 *Stu*I del or β-gal proteins bound ^{32}P-labeled linearized DNA (Fig. 2B, lanes 1–7). These data are consistent with previous studies showing that the C-terminus of Ku70 contains the Ku80-independent DNA-binding domain (42,43) and suggest that apoJ/XIP8 interacts with Ku70 at its C-terminal region to modify DNA binding.

Co-immunoprecipitation of apoJ/XIP8 and Ku70 in extracts from MCF-7 cells that overexpress apoJ/XIP8

ApoJ/XIP8 is a secreted glycoprotein (an intramolecular-cleaved, heavily glycosylated α/β polypeptide, linked by five disulfide bonds) found in numerous physiological fluids (44), which appears at ~40 kDa after SDS–PAGE under reducing conditions (1% β-mercaptoethanol) or at 80 kDa under non-reducing conditions (the five disulfide bonds remain intact) (44). A 60 kDa precursor of this protein (an uncleaved, less glycosylated form) was also identified in cells (45). An MCF-7 cell clone which stably overexpressed full-length apoJ/XIP8 was isolated (MCF-7-apoJ/XIP8; Fig. 3, lane 3) and analyzed along with cells expressing vector alone (Fig. 3, lanes 1 and 2). Log phase MCF-7-apoJ/XIP8 cells in RPMI medium with whole serum resulted in overexpression of the glycosylated precursor (60 kDa) and secretory (~40 kDa) forms of the apoJ/XIP8 protein (Fig. 3, compare lanes 1 and 2 to 3). Overexpression of this protein by stable transfection overcame the known testosterone repression (46,47). As expected, apoJ/XIP8 steady-state protein levels were elevated in log phase control MCF-7 cells treated with medium containing charcoal-stripped serum compared to log phase MCF-7 cells grown in medium containing whole serum (Fig. 3, compare lane 4 to 5). Stable transfection of MCF-7 cells with empty vector had little effect on basal apoJ/XIP8 expression (Fig. 3, compare lanes 1 and 2 to 4) in whole serum medium. apoJ/XIP8 overexpression did not change Ku70 or Ku80 protein levels (Fig. 3).

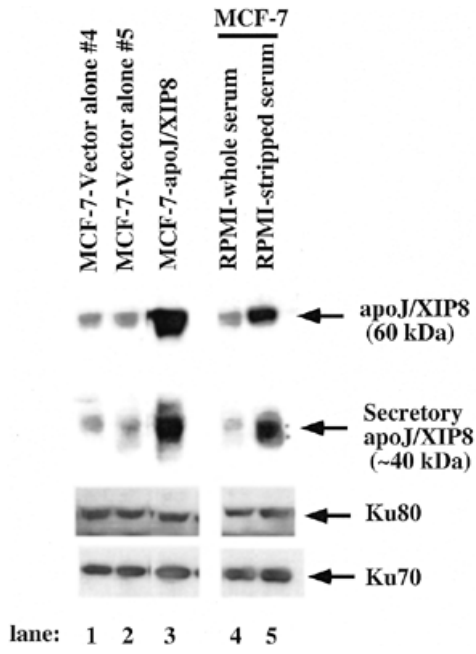

Figure 3. Analyses of stably transfected MCF-7 cells which overexpressed full-length apoJ/XIP8. Whole cell lysates were prepared from MCF-7-apoJ/XIP8 cells, which were stably transfected with CMV-driven full-length apoJ/XIP8 cDNA (lane 3), and compared to cells transfected with vector alone (lanes 1 and 2). Also, apoJ/XIP8 expression in MCF-7 cells treated for 3 days with RPMI medium containing whole serum (lane 4) or charcoal-stripped serum (lane 5) was analyzed by western immunoblot under reducing conditions as described in Materials and Methods. apoJ/XIP8 levels were detected using the anti-human clusterin (apoJ/XIP8) monoclonal antibody (41D), which recognizes the precursor (60 kDa) and secretory (~40 kDa, α and β intramolecular-cleaved peptides) forms of apoJ/XIP8 as indicated. Ku70 and Ku80 protein levels were detected using anti-human Ku70 (N3H10) and Ku80 (N9C1) monoclonal antibodies as loading controls.

Incubation of whole cell extracts (Fig. 4A, lane 8) from MCF-7-apoJ/XIP8 cells with apoJ monoclonal antibodies 1, 6, 11 or with polyclonal RαJβ antibody immunoprecipitated the 60 kDa form of apoJ/XIP8, which was detected using the anti-human clusterin (apoJ/XIP8) antibody (clone 41D) as described in Materials and Methods (Fig. 4A). The secretory form of apoJ/XIP8 was not observed. Ku70 was co-immunoprecipitated by apoJ monoclonal antibodies 1, 6 or by the RαJβ polyclonal antibody (Fig. 4A, lanes 2, 4 and 7), but not by apoJ monoclonal antibody 11 (Fig. 4A, lane 6), which only weakly immunoprecipitated apoJ/XIP8. In contrast, apoJ antibodies 5 and 10 were unable to immunoprecipitate apoJ/XIP8 and were also unable to co-immunoprecipitate Ku70 (Fig. 4A, lanes 3 and 5). Incubation of lysates with mouse IgG did not immunoprecipitate K70 or apoJ/XIP8 (Fig. 4A, lane 1).

Co-immunoprecipitation experiments were then performed in reverse fashion using antibodies directed against Ku70 to co-immunoprecipitate apoJ/XIP8. Rabbit anti-human Ku70 polyclonal antibodies 6 and 10 (36) immunoprecipitated Ku70, as well as co-immunoprecipitated 60 kDa apoJ/XIP8 (Fig. 4B, lanes 3 and 6). As before, the secretory form(s) of apoJ/XIP8 were not detected. The remaining Ku70 antibodies (2, 5, 7, 9 and 11) were unable to immunoprecipitate Ku70 and were also unable to co-immunoprecipitate apoJ/XIP8. A goat polyclonal anti-human Ku70 antibody (C19) was also able to co-immunoprecipitate Ku70 and apoJ/XIP8 (Fig. 4B, lane 8). The co-immunopreciptation

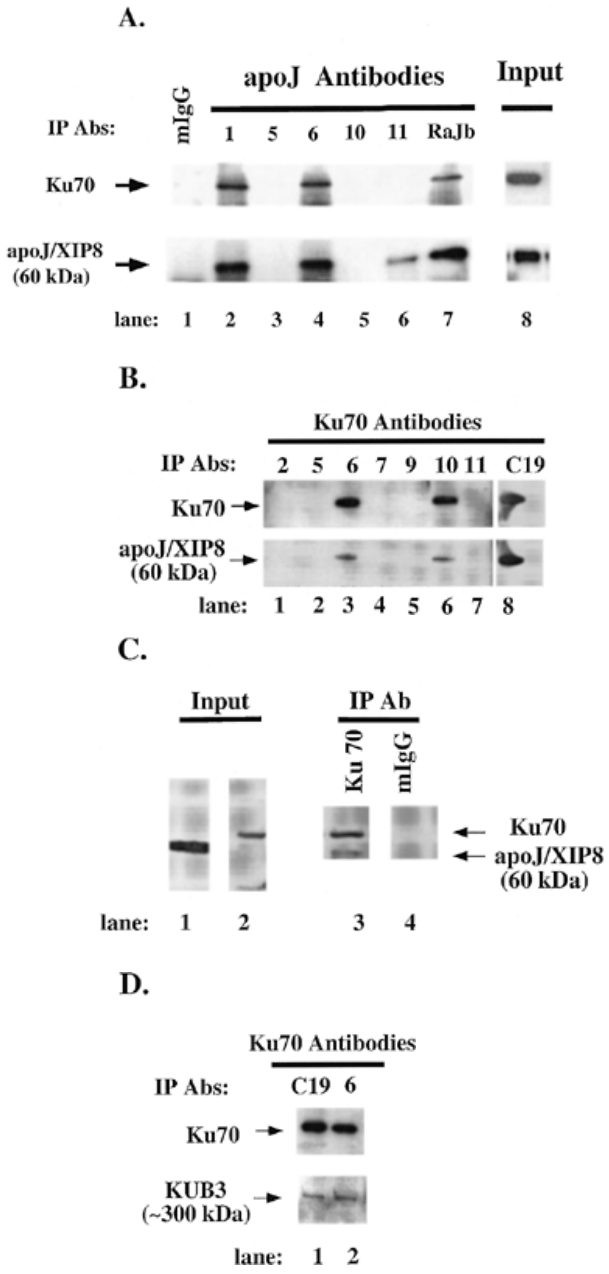

Figure 4. apoJ/XIP8 and KUB3 separately co-immunoprecipitate with Ku70. Whole cell extracts from log phase MCF-7-apoJ/XIP8 cells (Fig. 3, lane 3) were incubated with antibodies directed against the apoJ/XIP8 protein (antibodies 1, 5, 6, 10, 11 and RαJβ) (**A)** or with various Ku70 antibodies (2, 5, 6, 7, 9, 10, 11 and C19) (indicated as IP Abs) (**B)** as described in Materials and Methods. Immunoprecipitation with mouse IgG served as a negative control. Immunoprecipitates were separated by SDS–PAGE and examined by western immunoblot analyses for the presence of Ku70 (detected by the N3H10 antibody) or apoJ/XIP8 (detected by the 41D antibody) proteins. **(C)** Whole cell extracts from log phase HeLa cells, which express constitutive levels of apoJ/XIP8, were incubated with Ku70 N3H10 or mouse IgG antibodies to immunoprecipitate Ku70. Co-immunoprecipitation of apoJ/XIP8 was then examined. Only the ~60 kDa form of the apoJ/XIP8 protein was detected (lane 3). Mouse IgG did not immunoprecipitate Ku70 or apoJ/XIP8 (lane 4). The molecular weights of apoJ/XIP8 and Ku70 proteins were confirmed by western immunoblot analyses using whole cell extracts from MCF-7-apoJ/XIP8 cells (Input, lane 8 in A) or HeLa cells (Input, lanes 1 and 2 in C) in the same gel. **(D)** IMR-90 whole cell extracts were incubated with Ku70 rabbit polyclonal antibody 6 or goat polyclonal antibody C19 and analyzed for co-immunoprecipitation of KUB3 and Ku70 proteins. Only the ~300 kDa form of the KUB3 protein co-immunoprecipitated with Ku70.

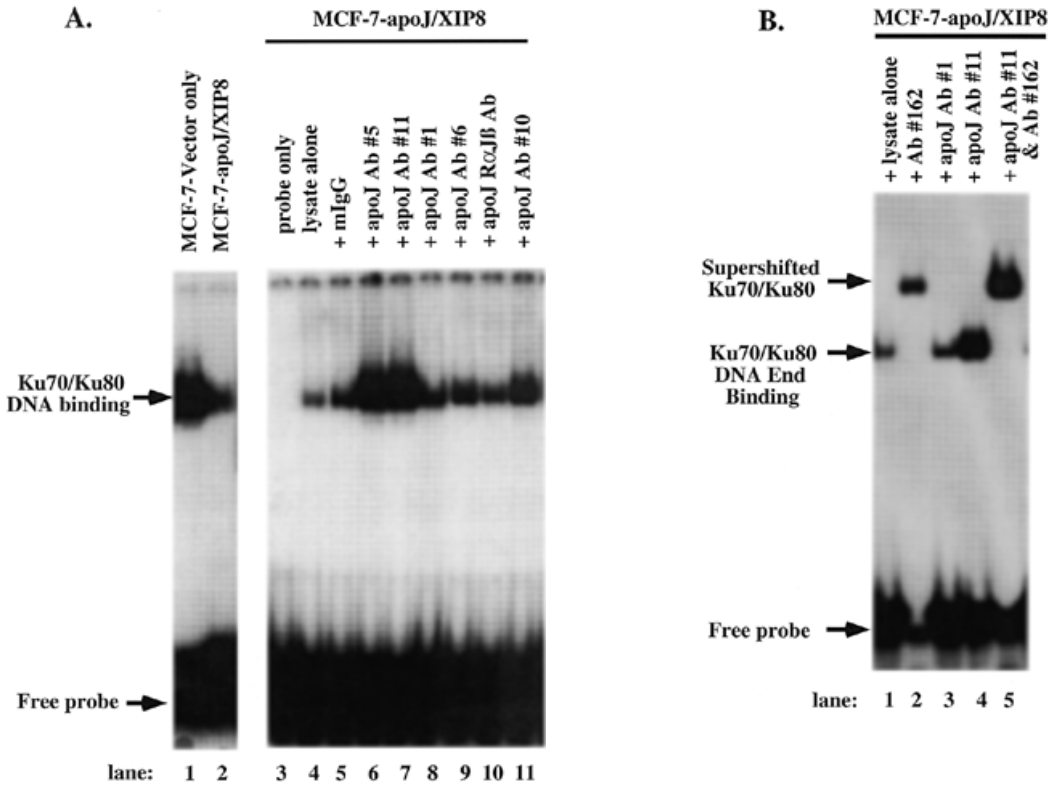

Figure 5. Overexpression of apoJ/XIP8 in MCF-7 cells decreased Ku70/Ku80 DNA end binding activity. Whole cell lysates from MCF-7-apoJ/XIP8 or MCF-7-Vector alone 5 (Fig. 3) were pre-incubated with ^{32}P-labeled linear DNA as described in Materials and Methods. Reactants were then separated by non-denaturing 5% PAGE. (**A**) Ku70/Ku80 DNA end binding activity from MCF-7-apoJ/XIP8 or MCF-7-Vector alone 5 are shown (lanes 1 and 2). Addition of apoJ antibodies to extracts from MCF-7-apoJ/XIP8 cells resulted in either no change (apoJ antibodies 1, 6 or RαJβ; lanes 8–10) or in increases (apoJ antibodies 5, 10 and 11; lanes 6, 7 and 11) in Ku70/Ku80 DNA end binding activity. Addition of mouse IgG (lane 5) did not affect DNA end binding compared to lysate alone (lane 4). **(B)** Antibody 162, which detects only the Ku70/Ku80 heterodimeric protein, was added to the binding reactions to supershift Ku70/Ku80 DNA-bound complexes (lanes 2 and 5). Addition of mouse anti-human apoJ monoclonal antibody 11 with anti-Ku70/Ku80 antibody 162 supershifted all increased Ku70/Ku80 DNA end binding activity (lane 5).

results above, in combination with yeast two-hybrid and far-western analyses, strongly suggest that Ku70 and apoJ/XIP8 [both glycosylated (60 kDa precursor) and non-glycosylated (bacterially expressed) forms] may associate *in vivo*.

Since certain HeLa cell lines are known to express constitutive levels of apoJ/XIP8 (47; Fig. 4C, lane 1), we examined whether this protein would co-immunoprecipitate with Ku70 under normal growth conditions without stable transfection of apoJ/XIP8. Incubation of whole cell extracts with monoclonal Ku70 antibody (N3H10) resulted in the co-immunoprecipitation of both Ku70 and the 60 kDa, but not the secretory form of apoJ/XIP8 (Fig. 4C, lane 3). Mouse IgG did not immunoprecipitate Ku70 nor apoJ/XIP8 (Fig. 4C, lane 4). Western blots using whole cell extracts from HeLa cells confirmed the molecular weights of apoJ/XIP8 and Ku70 (Fig. 4C, lanes 1 and 2). The co-immunoprecipitation results strongly suggested that Ku70 interacts with the 60 kDa, but not the secretory (~40 kDa), form of apoJ/XIP8.

Ku70–KUB3 interaction

Ku70–KUB3 protein interaction was also confirmed by co-immunoprecipitation. IMR-90 cells were used due to their relatively high constitutive KUB3 protein levels (Fig. 1). Co-immunoprecipitation of Ku70 and KUB3 was noted using either Ku70 goat polyclonal (C19) or Ku70 rabbit polyclonal 6 antibodies (Fig. 4D, lanes 1 and 2).

Overexpression of apoJ/XIP8 results in decreased Ku70/Ku80 DNA end binding activity

MCF-7-apoJ/XIP8 cells (Fig. 3, lane 3) had approximately 5-fold lower DNA end binding activity compared to MCF-7-Vector alone 5 cells (Fig. 3, lane 2) (compare DNA end binding in Fig. 5A, lane 2 to 1). Ku70 and Ku80 protein levels did not change due to apoJ/XIP8 overexpression (Fig. 3). Addition of apoJ antibody 5 or 11 and, to a lesser extent, antibody 10, increased Ku70/Ku80 DNA end binding compared to addition of non-specific mouse IgG antibody or lysate alone (Fig. 5A, compare lanes 6, 7 and 11 to 4 and 5, respectively); these antibodies did not co-immunoprecipitate Ku70 and apoJ/XIP8 complexes (Fig. 4A). Interestingly, addition of apoJ antibodies 1, 6 and RαJβ did not affect Ku70/Ku80 DNA end binding activities (Fig. 5A, lanes 8–10); these antibodies co-immunoprecipitated Ku70 and apoJ/XIP8 (Fig. 4A).

Addition of mouse anti-human Ku70/Ku80 antibody (Ab 162, which specifically detects the Ku70/Ku80 heterodimer; 35) supershifted the DNA end binding activity from MCF-7 cell lysates (Fig. 5B, lane 2). When the Ku70/Ku80 Ab 162 and apoJ 11 antibodies were co-administered, the enhanced Ku70/Ku80

end binding activity in MCF-7-apoJ/XIP8 cell extracts was supershifted (Fig. 5B, lane 5). These results suggested that apoJ/XIP8 can modify (i.e. partially prevent) Ku70/Ku80 DNA end binding activity, which is consistent with its binding to the C-terminus of Ku70 (Table 1 and Fig. 2). Addition of apoJ/XIP8 antibodies 5 or 11, which failed to co-immunoprecipitate Ku70 (Fig. 4A), apparently dissociated apoJ/XIP8 from Ku70, leading to increased Ku70/Ku80 end binding activity (Fig. 5).

IR-inducible apoJ/XIP8 modifies Ku70/Ku80 end binding activity

Induction of apoJ/XIP8 in MCF-7 cells after IR was confirmed at the protein level by western immunoblot analyses (Fig. 6); time course and dose–response studies will appear elsewhere (C.-R.Yang, E.Odegaard, G.Zhu, B.Aronow, J.K.Harmony and D.A.Boothman, submitted for publication). Induction of apoJ/XIP8 (both the 60 kDa and secretory forms) in MCF-7 cells 3 days after 10 Gy was shown. In contrast, Ku70 and Ku80 protein levels remained unchanged 3 days post-IR (Fig. 6, lanes 1 and 2). The same extracts from irradiated and non-irradiated MCF-7 cells were then examined for DNA end binding activity as described in Figure 5. As observed with transfected MCF-7-apoJ/XIP8 cells (Fig. 5), decreased levels of Ku70/Ku80 DNA end binding activity were also noted (Fig. 6, lanes 4 and 5). Decreased DNA end binding activity correlated with peak levels of apoJ/XIP8 appearing in irradiated MCF-7 cells 3 days post-irradiation. DNA end binding activity was not affected in MCF-7 cells at 0, 1 or 2 days post-irradiation (data not shown), at times when apoJ/XIP8 protein levels were not elevated. Since elevated levels of apoJ/XIP8 (by IR treatment or overexpression) lowered Ku70/Ku80 DNA end binding activity and this activity was required for DSB repair and survival in mammalian cells (13), we tested whether MCF-7-apoJ/XIP8 cells were more sensitive to IR than MCF-7-vector cells. MCF-7-apoJ/XIP8 cells were not more radiosensitive compared to MCF-7-Vector alone cells as measured using colony forming assays (data not shown). The dramatic IR inducibility of the endogenous apoJ/XIP8 gene in MCF-7 cells (Fig. 6) appears to be responsible for the lack of radiosensitivity of MCF-7-apoJ/XIP8 cells. These data demonstrate that elucidating the role of apoJ/XIP8 in cell radiosensitivity must be performed in apoJ-deficient cells.

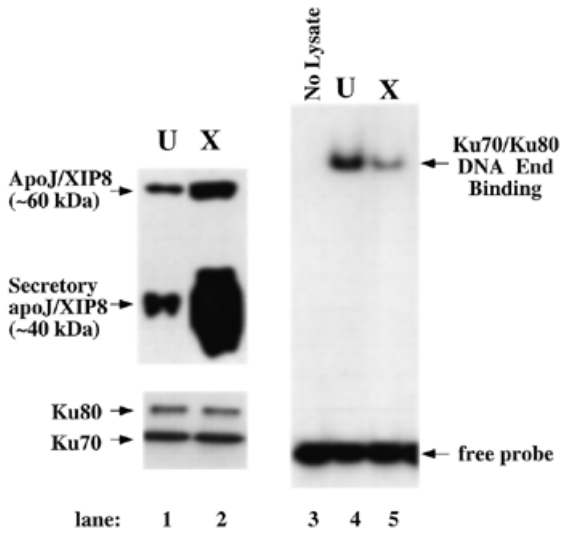

Figure 6. Endogenous apoJ/XIP8 protein was induced after IR treatment. Whole cell extracts from unnirradiated (U) or IR-treated (X-rays, 10 Gy, 3 days post-irradiation) MCF-7 cells were analyzed for apoJ/XIP8, Ku70 and Ku80 protein expression and Ku70/Ku80 DNA end binding activity as described in Materials and Methods. The 60 kDa and secretory forms of the apoJ/XIP8 protein were induced, whereas Ku70 and Ku80 levels remained unchanged.

DISCUSSION

To our knowledge, we are the first to successfully perform a yeast two-hybrid cDNA screen using the Ku70 protein as 'bait' and isolate KUBs other than Ku80. Our other attempts to clone proteins associated with Ku80 or Ku70/Ku80 (simultaneously co-expressed) failed (data not shown). Co-expression of Ku70 and Ku80 resulted in transactivation of reporter genes (His^+, β-gal^+), making yeast two-hybrid screening impossible. Attempts to use Ku80 as 'bait' failed for unknown reasons. Using Ku70 we were, however, able to isolate the Ku80 cDNA, a well-known partner of Ku70 (17). We also isolated two fairly large transcripts, KUB3 and KUB4, which contained no homology to known genes in GenBank; these genes lacked homology to DNA-PK_{cs} or the related ataxia telangiectasia (ATM) genes and contained no unique sequence motifs. Northern blot analyses detected single transcripts in excess of 10 kb for KUB3 and KUB4 in various human cells (Fig. 1). DNA sequence and limited expression data suggested that KUB3 and KUB4 represented two separate transcripts, but isolation of full-length cDNAs will be required for verification. The interaction between KUB3 and Ku70 was confirmed by co-immunoprecipitation analyses. Confirmation of the interaction between KUB4 and Ku70 *in vivo* must await additional sequence information and the generation of polyclonal antiserum. Further research will be required to elucidate the function(s) of these proteins in DSB repair, telomere maintenance, senescence or injury responses after IR treatments in mammalian cells. The large sizes of these transcripts will probably require extensive cloning procedures, as required for the DNA-PK_{cs} and ATM genes (48,49). We concentrated, therefore, on the apoJ/XIP8 protein, which was isolated from the screen described in Table 1 along with Ku80.

We are the first to demonstrate that Ku70 interacts with the apoJ protein (40), also known as clusterin (41) and TRPM-2 (41), among many other names (50). This gene is a known 'marker for apoptosis' (30,51–53). We previously isolated apoJ as X-ray-induced transcript no. 8 (xip8) by differential hybridization (38). Induction of apoJ/XIP8 in MCF-7 cells after IR was confirmed at the protein level by western immunoblot analyses (Fig. 6). To our knowledge this is the first stress-inducible protein which associates with the DNA-PK DSB repair protein complex.

We demonstrated that the 60 kDa precursor (endoplasmic reticulum-targeted) form of apoJ/XIP8 can associate with the Ku70 protein, which is presumably nuclear. The secretory form of apoJ/XIP8 did not associate with Ku70 in co-immunoprecipitation analyses (Fig. 4). We hypothesize that the secretory form of the apoJ/XIP8 protein probably does not interact with Ku70 due to its extensive post-translational glycosylation. Recent reports have suggested that Ku70/Ku80 and DNA-PK_{cs} (i.e. the entire DNA-PK complex) may interact with the EGF receptor, near the cell membrane (54). Other immunocytochemical data using a variety of Ku70 antibodies have also demonstrated that the Ku70 protein is present in the cell membrane of HeLa cells (36). It is, therefore, possible that apoJ/XIP8 may interact with

Ku70 in the cell membrane and that this interaction may initiate complex signal transduction processes which trigger cell death, possibly by apoptosis. Further research using apoJ/XIP8 genetically deficient cells will be required to dissect these potentially diverse signaling events in the cell.

The only evidence that apoJ/XIP8 may directly affect DSB repair is that its overexpression (either by stable transfection or IR inducibility) caused a decrease in Ku70/Ku80 DNA end binding activity when whole cell extracts were used (Figs 5 and 6A). These data alone are, however, insufficient to conclude that apoJ/XIP8 affects DNA repair. The inhibition of Ku70/Ku80 DNA end binding activity (a Ku80-dependent activity) (43) in overexpressing transformants or in IR-treated MCF-7 cells (Figs 5 and 6A) could well be due to steric hindrance and prevention of both Ku80-dependent and -independent DNA end binding activity. Further research into the exact protein–protein interaction domains between Ku70 and apoJ/XIP8 and any possible altered function of DNA-PK by apoJ/XIP8 is ongoing in our laboratory and will be reported separately.

Conversely, the fact that overexpression of apoJ/XIP8 did not radiosensitize stably transfected MCF-7 cells also does not mean that this protein does not affect latent DNA repair or delayed repair/apoptotic intracellular reactions. The dramatic IR inducibility of this protein suggests, in fact, that the endogenous gene may be dominant over any attempts to overexpress the protein; we were unable to increase expression of this protein (by transient or stable transfection) above that observed after IR treatment. The role of apoJ/XIP8 in radiosensitivity must, therefore, await the generation of genetically deficient (e.g. knockout) animals. Further research will be required to prove that it is the specific interaction of apoJ/XIP8 with Ku70 that triggers a death signal in IR-treated cells.

The apoJ/XIP8 protein has been cloned from many diverse biological systems. This protein has been given numerous names, including clusterin, apolipoprotein J (apoJ), SGP-2, TRPM-2, SP-40,40, CLI, pADHC-9, XIP8 and others (reviewed in 50). The protein may play numerous roles in various biological systems, including (i) reproduction (clusterin and SGP-2); (ii) lipid transport and heart injury responses (apoJ); (iii) complement regulation (CLI and SP-40,40); (iv) central nervous system disorders and developmental processes (pADHC-9); (v) apoptosis (TRPM-2); and (vi) cell–cell interactions (clusterin) (reviewed in 50). Increased expression of clusterin has been observed in aging (55–57) and in various diseases, such as neurodegenerative diseases (Alzheimer's disease and scrapie), renal diseases (glomerulonephritis), atherosclerosis and in some cancers (reviewed in 50). We theorize that the association between Ku70 and apoJ/XIP8 may correlate with the pathophysiology of many apoJ/XIP8-related diseases and may provide a useful clinical marker of injury responses, including radiation injury to normal and tumor tissues. Thus, investigation of stress-inducible apoJ/XIP8 responses has the potential to increase our understanding of the interrelations of cell death, tissue responses to injury, lipid transportation, spermatozoa maturation, complement regulation, neurodegenerative disorders and aging.

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